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A. Fluorometric Investigation of
Early Chicken Embryo Development

University — Université

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Degree for which thesis was presented — Grade pour lequel cette thèse fut présentée

Masters of Science

Year this degree conferred — Année d'obtention de ce grade

Fall 1979

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THE UNIVERSITY OF ALBERTA

A FLUOROMETRIC INVESTIGATION OF EARLY

CHICKEN EMBRYO DEVELOPMENT

by



DAVID L. DEMOREST

A THESIS

SUMMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

IN

DEPARTMENT OF ZOOLOGY

EDMONTON, ALBERTA

FALL, 1979

THE UNIVERSITY OF ALBERTA

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The undersigned certify that they have read, and recommended to the

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to my wife joanne

ABSTRACT

Progressive changes in embryonic substances during the first week of chick embryo development were studied by fluorospectrophotometric analysis. The fluorescence patterns characteristic of each developmental stage were recorded from whole embryo homogenates between three days and six days of developmental age. Changes in the spectral pattern were noted and the precise time in development pinpointed. Differential fractionation techniques were employed in order to define the sub-cellular location of the fluorophores involved. Finally, an attempt was made to characterize and identify the fluorescent substances.

In order to correlate changes in the fluorescence pattern with developmental age, it was first necessary to define the developmental stage of individual embryos. However, the possibility of fluorophore decomposition and/or the production of artifactual fluorescence made it essential to minimize exposure of the embryos to light and oxygen. Thus it became necessary to devise a rapid but accurate means of recording and identifying developmental stage.

In hope of finding such a method, the relationship between several growth parameters and the stage of development was examined. A cranial length measurement was found to be highly correlated with developmental stage over the period of development from three to six days. This correlation remained unchanged by minor differences in incubation temperature, which sometimes alters such a relationship. Thus, the use of the cranial length, which is easily measured from photographs, presents a method by which to determine the developmental stage of individual embryos, quickly and accurately.

Fluorometric analysis revealed that three major emission peaks

dominate the fluorescence pattern during the first week of development. Two of the peaks, 350nm and 520-525nm, merely increase in relative fluorescence. The fluorophores responsible for these two peaks were later determined to be, respectively, tryptophane residues in soluble protein, and riboflavin or its derivative thereof.

The third region of emission was found to undergo a pronounced red-shift in emission peak from 430nm at stage 22 to 470nm by stage 25, again accompanied by a gradual increase in relative fluorescence. Such a shift indicates a specific change at the molecular level, at that stage of development. This prompted an investigation into the identity of the fluorophores involved and into the cause of the shift.

Fractionation revealed that the majority of the 430-440nm and 470nm fluorescent components in the three-day and six-day homogenates were found in the soluble-protein fraction. This suggests that the fluorophores are freely soluble or that they are bound to a "carrier" molecule.

Attempts to identify the 470nm emission component were unsuccessful, due to the instability of this peak. During isolation attempts, the peak shifted to a peak at 440nm. However, the resulting spectral pattern mimicked that of the younger embryo. This result suggests that a single type of fluorescent component is involved in the original spectral shift. Thus, further investigation was concentrated on isolation of the component responsible for the 440nm emission peak.

Solubility characteristics and response to specific enzymatic digestions revealed that the 440nm emission component is associated with a variety of binding components. Again, these results suggest that the spectral shift seen between stage 22 and stage 25 is due to

the change in the type of binding components of a single type of fluorophore. Upon binding to a different "carrier," the molecular environment of the fluorophore may be altered in a manner which affects the spectral characteristics.

Unfortunately, upon release from the "carrier," the 440nm emission substance again became unstable, frustrating further efforts at isolation and identification. It will be necessary to purify and characterize the "carrier" fluorophore complex before identification can be made and determination as to whether a single type of fluorescent substance is involved. It should then be possible to proceed with investigations into the biological significance of the spectral shift reported in this study.

ACKNOWLEDGMENTS

I would like to thank Dr. J. K. Lauber, Dr. M. Kato, and Dr. S. I. Igarashi for providing the very valuable information on their own observations of chick embryo fluorescence. The major premise of this thesis derives from these earlier works.

I would like to thank Dr. J. K. Lauber for providing the facilities for the biological portion of this study, and for her critical analysis of this manuscript.

I would like to thank Dr. S. I. Igarashi for providing the facilities and resources to carry out the biochemical portion of this study. I appreciate also the long hours of discussion as to how to approach this and other biological problems, and I am grateful for his critical analysis of this manuscript.

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LIST OF ABBREVIATIONS

SME	- β -mercaptoethanol
PMSR	- Phenylmethanesulfonyl fluoride
TCA-	- trichloroacetic acid
Tris-	- tris (hydroxymethyl) amino methane
DEAE-	- diethyl amino ethyl
RF	- riboflavin
FAD	- flavin adinine dinucleotide
FMN	- flavin mononucleotide
x g	- times gravity
M	- molar
gm	- grams
mg	- milligram
μ g	- microgram
l	- liter
ml	- milliliter
mm	- millimeter
m	- micrometer
sec	- second
min	- minute
hrs	- hours
$^{\circ}$ C	- degrees Centigrade (Celsius)
Cu/Con	- copper/constantan
H-H.stage	- Hamburger and Hamilton stage
C.L.	- cranial length
MB	- middle brain
FB	- fore brain

E - eye
WB - wing bud
LB - leg bud
S.D.M. - standard deviation from the mean
S.E.M. - standard error of the mean
ex - excitation
em - emission

CHAPTER 1

GENERAL INTRODUCTION

The central problem in the study of embryonic morphogenesis concerns the mechanisms involved in determination, initiation, and synchronization of the cytological, histological, and morphological changes which occur as development proceeds. Interactions between cell population, like and unlike, and between the cells and their microenvironment are intimately involved in these control mechanisms (for a review, see Fleischmajer and Billingham, 1968; Brunner, 1977). Spemann and Mangold (1924) were the first to describe the phenomenon of primary induction; they observed that, during gastrulation in the amphibian embryo (Triturus taeniatus), the dorsal lip of the blastophore exerts an inductive effect on the overlying ectoderm, causing it to form the neural plate. Later, Waddington (1933) demonstrated that Hensen's node plays a similar role (acting as the primary inducer) in the development of the chick embryo (reviewed by Leikola, 1976). Many cases of secondary and tertiary induction are now known to be required for cyto-differentiation and morphological change during organogenesis (Fleischmajer and Billingham, 1968). A few of the morphological changes which require specific inductive interactions are kidney tubule development (Gorbstein and Dalton, 1957), salivary gland formation (Gorbstein, 1953), and limb bud formation (Stark and Searls, 1973; Searls and Janner, 1973).

Spemann's original observation prompted many investigations aimed at the isolation and identification of unique "inducer" substances produced by inducer cells (review by Holtfreter, 1968). Although a few natural inducer substances have been isolated during the past fifty years (review by Tiedemann, 1976), a variety of foreign tissues (Takata

and Yamada, 1960; Kawakami, 1976), heat-killed tissues (Bautzmann, et. al., 1932), ions such as Na^+ , Ca^{+2} , K^+ (review by Holtfreter, 1968; Brunner, 1977), and even treatment with methylene blue (Waddington, et. al., 1936) have been found to mimic induction. Holtfreter (1968) noted that the use of these artificial inducers has provided valuable clues about the process of induction. However, the molecular mechanisms of induction within the embryo itself are still not understood.

In the past two decades much evidence has been accumulated on the physical and chemical effects of the extracellular matrix on differentiation and morphogenesis (Manasek, 1975; Manasek, 1976a). For example, morphogenic movements in heart formation are apparently directed by regional accumulation of glycoprotein in the basal laminae (Manasek, 1976b). The extracellular matrix may also serve in a permissive or modulator role in induction: matrices containing collagen, glycosaminoglycan, and glycoprotein are found at many sites of induction (Koch and Gorbstein, 1963; Kosher and Lash, 1974; Newsome, 1976). The glycosaminoglycans and glycoproteins may react with specific receptors and the cell membrane of the induced cell, activating certain metabolic pathways (Nevo and Dorfman, 1972). The matrix may influence the microenvironment surrounding the receptor cells by concentrating ions, or by binding enzymes, nutrients, and/or other substances necessary for cell proliferation and differentiation (Newsome, 1976). Therefore, changes in the composition of the matrix during development may play a passive role in the temporal programming of differentiation and morphogenesis (Newsome, 1976).

In line with the influences of the extracellular matrix noted above, Brunner (1977) has recently presented an hypothesis as to how sequential

changes in the cellular microenvironment may direct the process of differentiation. He proposed that the cell membrane receptors act as mediators between environmental signals and genome expression, in a process termed "membrane impression". According to this hypothesis, signals from the external environment are received by specific receptors in the cell membrane. These signals are transduced and transmitted through intracellular informational channels, termed an "effector network" by Seglen (1976), to the genome, and ultimately influence gene expression. The result is that, in anticipation of new environmental signals, new receptors are prepared along with the expression of a new cell phenotype. Brunner (1977) suggested that, in order to understand the control of differentiation (and thereby morphogenesis), more must be understood about the composition of the cellular microenvironment, the type of receptors available, and the "network" of enzymatic and chemical reactions which ultimately lead to a change in the pattern of gene expression.

Nutritional studies, both in vivo and in vitro, have demonstrated that specific vitamins, proteins, and other nutritional factors are required at various stages of development (review by Romanoff, 1967). Enzymatic studies have provided clues as to the metabolic processes utilized by the developing embryo to meet the energy requirements for rapid cell proliferation and morphological change (Klein, 1976; Coffin and Hall, 1974; Seltzer, 1975). Enzymatic studies have been used to examine the state of differentiation of certain tissues. This approach provides a sensitive means of detecting cytological changes during development (Medoff, 1967; Murzullo and Desiderio, 1972). Also, enzymatic studies have provided evidence as to the role of metabolites such as

cyclic AMP and cyclic GMP in the intracellular control of differentiation (McMahon, 1974; Culter and Rodan, 1976). Drugs, such as 5-bromodeoxyuridine, 5-fluorodeoxyuridine, and actinomycin D, interfere with transcription and translation. These have been used to examine the role which Holtzer (1976) terms the "quantal cell division" in the control of differentiation.

Another approach to the study of the control of development and morphogenesis has been to examine the effect of environmental factors on normal embryonic development; minor variations from the optimal incubation conditions have pronounced effects on normal growth and development (review by Landauer, 1961). Several groups of investigators have reported that chicken and turkey eggs, illuminated under various light regimes during incubation, hatch earlier than eggs incubated in the dark (Shutze, et.al., 1962 ; Lauber and Shutze, 1964; Cooper, 1972). Siegel, et. al., (1969), Isakson et. al., (1970) and Lauber (1975) found that acceleration of embryonic growth due to light exposure is in fact detectable within the first week of incubation. Lauber (1975) suggested that one possible approach to understanding the mechanisms involved in the light acceleration of development would be to identify the photoreceptor. Many fluorescent substances are quite unstable upon exposure to light, making them prime candidates for the photoreceptor. Therefore, Lauber and Kato (personal communication) conducted a preliminary fluorometric examination of the first week of development of the chick embryo. Groups of embryos of approximately the same body wet weight, between day three and day six of incubation, were dissolved in 75 percent sulfuric acid, and the

fluorescence spectra recorded. This examination revealed that several regions of emission dominate the spectral pattern, one of which shows a red-shift as development proceeds.

However, sulfuric acid is known to induce intense fluorescence in various biological compounds (review, see Morton, 1976). Therefore, Igarashi (personal communication) followed Lauber's study by examining the spectral patterns obtained from embryos of the same developmental age utilized in the earlier study, but homogenized in a buffer containing phenylmethanesulfonyl fluoride, 0.25 M sucrose and ρ ME. This buffer maintains at least some enzymatic activity and minimizes leakage of mitochondrial and nuclear substances (Igarashi, personal communication). Igarashi's study confirmed the earlier observation; three major regions of fluorescence were noted, the middle region shifting to the red as development proceeds. In addition, Igarashi (unpublished data) fractionated the homogenate of chick embryos by differential centrifugation in an attempt to identify the sub-cellular location of these fluorescent substances. The major amount of fluorescence at each of the three emission peaks was found in the post-mitochondrial fraction, suggesting that the fluorophores are either free in the serum or cytosol, or bound to a soluble matrix. These fluorophores may or may not be involved in the photo-acceleration of development. It is probable, however, that the spectral shift is an indication of metabolic changes as development proceeds, or that it may be the result of a change in the microenvironment of a certain fluorophore during the first week of development. Therefore, a further study of embryonic fluorophore was warranted.

The purpose of this thesis was to expand upon the fluorometric investigations conducted by Lauber, Kato and Igarashi, and to examine the

embryos homogenized in a buffer solution. However, a slightly different buffer solution, buffer "B" (Igarashi, 1967), was utilized. This buffer did not contain PMSF, which is fluorescent and would interfere with our fluorometric observations. The spectra were recorded from homogenates of individual embryos of Hamburger-Hamilton stage 16 to 30 in order to define the precise spectral pattern exhibited at each stage. This allowed us to pinpoint exactly when during development any changes occur, in particular the pronounced spectral shift observed in earlier investigations. Furthermore the sub-cellular location of the embryonic fluorophores was determined. An attempt was then made toward isolation and identification of these embryonic fluorophores, in hope of elucidating the role of these substances in morphological and cytological changes which occur at this point in development.

CHAPTER 2

MATERIALS AND METHODS

2-1. Materials:

DEAE-cellulose (exchange capacity 1.20 meg/ml) was purchased from Whatman (Clifton, N.J.). Phosphocellulose (exchange capacity of 0.99 meg/ml) was purchased from Schleicher and Schuell, Inc. (New Haven, Conn.). Sephadex G-10 (particle size 40-120) was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Sucrose of the ultra pure quality, special enzyme grade, was purchased from Schwartz/Mann Co. (Orangeburg, N.Y.). Acridine orange, Pronase (Streptomyces griseus protease), and α -chymotrypsin (bovine pancreas) were purchased from Calbiochemical Co. (Los Angeles, Cal.). Riboflavin was purchased from Bio-Rad Laboratories (Mississauga, Ontario). Trizma base, flavin mononucleotide, flavin adenine dinucleotide, hyaluronidase (bovine testis), lipase (hog pancreas), L-tryptophane, and L-tyrosine were purchased from Sigma Chemical Co. (St. Louis, Mo.). All other chemicals were of reagent grade purchased from the J.T. Baker Chemical Co. (Phillipsburg, N.J.) and were used without further purification. Eggs, from a stock of White Leghorn hens studied in this laboratory for many years, were obtained from the University of Alberta poultry farm (Edmonton, Alberta).

2-2. Buffering System and Fixative:

The following buffer-salt solutions were used as required: Buffer "B": 10 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 50 mM KCl, 6 mM β -mercaptoethanol (Igarashi, 1967).

Buffer "K": (pH 7.0), 30.5 mM K_2HPO_4 , 19.5 mM KH_2PO_4 , 1 mM β -mercaptoethanol (Igarashi, 1969).

The following solution was used to fix embryos: Bouin's liquid:

75% saturated picric acid, 25% commercial formalin, 5% glacial acetic acid (Gabe, 1976).

2-3. Methods:

(i) Preincubation Treatment of Eggs: Only eggs collected from the afternoon lay were utilized, to minimize initiation of development prior to the beginning of incubation. In each experiment four dozen eggs, weighing from 50 to 60 grams each, were randomly arranged in rocker trays. The eggs were brought to room temperature three hours prior to the stage of incubation and placed in a pre-warmed incubator at approximately the same time of day in each experiment.

(ii) Incubation Conditions: (a) Incubation conditions for the determination of the relationship between H-H. stage, body wet weight, and cranial length: A David Bradley forced air incubator (model 400, Brower Mfg. Co., Quincy, Ill.) was utilized. Eggs were turned manually twice daily throughout the incubation period. A continuous recording of air temperature was made in a modified Rustrak recorder model 91/131 with a thermistor air probe (#TP 2002, Rustrak Inst. Inc., Manchester, N.Y.). The temperature was calibrated with a Conmark electronic thermometer 1642 BPU, type 1642 (International Rectifier Co., Scarborough, Ontario), equipped with P-38 Cu/Con (copper/constantan) thermocouples (Leeds and Northrup Co., Calgary, Alberta). The mean air temperature was maintained at $37.5^{\circ}\text{C} \pm 0.3^{\circ}\text{C}$ over the first seven days of development; however, due to the nature of the heating system there was a rapid fluctuation of approximately $\pm 0.6^{\circ}\text{C}$ around this mean every sixty seconds. The mean air temperature was monitored by placing the air probe in the center of the incubator at the level of the eggs. The relative humidity was maintained at 50 to 60 percent as measured with a humidity probe (Model

15-3001, Hydrodynamics Co., Silver Springs, Maryland).

(b) Incubation conditions utilized to examine temperature effects and also to obtain embryonic material for fluorometric analysis: A National Appliance Co. incubator (model 3221, Heinicke Co., Portland, Oregon) was selected for use here. The air temperature, maintained by a water-jacket heating system, varied $\pm 0.15^{\circ}\text{C}$ around the mean every six hours. For the first six days of incubation the mean air temperature was maintained within $\pm 0.5^{\circ}\text{C}$ of the set incubation temperature of each experiment. Adequate ventilation was assured by a pre-heated air flow of 12 liters/minute. Relative humidity was maintained at 60 percent by filling the bottom of the incubator with water.

Temperature within the egg was monitored by inserting a Cu/Con P-38 thermocouple into a small hole drilled at the blunt end of the egg. The holes were sealed immediately with hot paraffin and the thermocouple held in place with a small piece of Scotch tape. In each experiment, four eggs equipped with thermocouples were distributed randomly in the incubator among those set. The thermocouples were connected to the Conmark electronic thermometer equipped with a multi-channel selector unit (model 1694F, International Rectifier Co., Scarborough, Ontario). The egg air space temperatures were checked manually every twelve hours. Also, a continuous record of incubator air temperature was maintained with a Rustrak recording system described earlier.

(iii) Treatment of Embryos:

(a) Method of removing, staging, weighing, and photographing each embryo: After incubation for the desired period, eggs were removed from the incubator and cracked into a glass bowl. The embryo was cut free from the extraembryonic membranes with scissors, and lifted out with a

spatula. The amniotic sac was removed with forceps, and the embryo was washed with 0.9 percent saline to free yolk substances still adhering. Each embryo was placed in a small weighing boat and the excess fluid was drawn off with a slip of filter paper. The wet weight was determined with a Federal Pacific precision torsion balance, model LG 250 mg capacity (Northboro, Mass.).

The embryo was photographed under the following conditions: after removal of the extraembryonic membranes and the excess yolk, each embryo was placed on its left side in a small plastic petri dish. The dish was set on a light box (model 12-120, I.R.I. Co., Cheltenham, Pa.). Two optic fiber light guides, FD3A-3, were directed toward the embryo from an incandescent source (Iota-Cam fiber optic corp., Wakefield, Mass.). A picture of the embryo was taken with a Nikon F camera with a 20 mm F/5.6 lens. The camera was mounted on a Nikon F repro-copy stand PF (Nippon Kogaku, Tokyo, Japan).

(b) Treatment of the embryos used to establish the staging system: When preparing embryos for use in establishment of a staging system, three eggs were sampled every twelve hours from 72 to 168 hours of incubation. Each embryo was removed from the egg, weighed, and photographed in the manner described above. The H-H. stage of each embryo was then carefully determined under a Zeiss Stereozoom III dissecting microscope (Carl Zeiss Canada Ltd., Toronto, Ontario) with total magnification of 1x to 4x. The embryos were then placed in Bouin's fixative and re-examined at a later time, to confirm the staging decision.

(c) Treatment of embryos for the determination of temperature effects and for use in fluorometric analysis: Embryos used for these studies were treated slightly differently from the treatment described in (a) and

(b). Twelve eggs each day were sampled on the third, fourth, fifth, and sixth day of incubation in each of the four temperature experiments. Each embryo was removed from the egg, weighed, and photographed as described earlier. In order to ensure complete removal of yolk substance, each embryo was washed five times with clean 0.9 percent saline. Care was taken to minimize the exposure of embryos to light, thereby preventing photo-decomposition of embryonic substances which might be of interest in future studies. Embryos were frozen in liquid nitrogen within two minutes after removal from the egg. The frozen embryos were placed in individual vials and stored in the dark at -20°C for later analysis.

(iv) Measurement of Cranial Length: The cranial length of each embryo was measured on the photographic negatives. This is illustrated in Fig. 1 of Chapter 3. All measurements were corrected to the value of 1x magnification.

(v) Preparation of Embryo Homogenates Utilized in Fluorometric Analysis: Individual embryos were homogenized in 1 ml of buffer "B" at pH 7.8, with a glass homogenizer. The homogenizer was then washed with 1 ml of buffer "B", which was then added to the original homogenate. The solution was clarified by centrifugation at $12,000 \times g$ for 10 minutes at 4°C in a Beckman J-21 refrigerated centrifuge (Beckman Instrument Co., Palo Alto, Cal.). The post-nuclear supernatant was drawn off and brought to a final volume of 4 ml with buffer "B" and frozen. The entire procedure was performed in dim light, and the vials containing each supernatant were wrapped in aluminum foil and placed on ice to minimize the possibility of photo-decomposition.

(vi) Fluorometric Analysis: A Baird-Atomic "Fluorispec" (Model SF-100,

Baird-Atomic Corp., Newark, N.J.) equipped with a Houston X-Y recorder (Model 2000), Bausch and Lomb Corp., Rochester, N.Y.) was utilized to examine and record the spectral patterns. The range of intensities detectable by this instrument are: an upper limit equivalent to the fluorescence exhibited by a 0.1 mM solution of acridine orange in double distilled water; a lower limit equivalent to the fluorescence exhibited by 0.0001 mM solution when excited at 470 nm and the emission read at 530 nm. All spectral recordings were calibrated to a photometer sensitivity of fine gain at 10 and course gain at 300, with the X-Y recorder set of 10 mv/inch. At this sensitivity a 0.1 mM acridine orange solution yields a relative fluorescence value of 13.

The temperature of the water-jacketed cuvette chamber was maintained at $19.0^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$, with a Haake circulator (Model F-4391, Haake Inst., Berlin, West Germany). The spectra were always recorded from the longer wavelength to the shorter wavelength in order to minimize photo-decomposition of the fluorophores of interest. As an additional precaution against photoadduct formation, each embryonic sample was examined only once. In order to quantitate the fluorescence intensity, a 0.01 mM solution of acridine orange in double distilled water was used as a standard in each spectral recording.

(vii) General Fluorometric Survey of Embryonic Development: Embryos were taken from an experimental group incubated at 37.5°C . Embryos incubated for three to six days were staged on the basis of cranial length and H-H. stage. The post-nuclear fraction from each embryo was prepared and examined fluorometrically as already described. The major peaks of excitation and emission wavelengths were recorded and standardized (Chapter 4 section iv).

(viii) Examination of the Fluorescence Pattern from Egg Yolk: Samples of yolk were prepared from the unbroken yolk of an unincubated egg, and from eggs incubated from three to six days. The samples were placed in a 1 ml plastic vial, covered with aluminum foil, and frozen immediately. Two hundred mg portions of each yolk sample were thawed and homogenized separately, as previously described for whole embryos. The resulting yolk supernatants, each suspended in 4 ml of buffer "B", were further diluted eight times with buffer in order to reduce light scattering, and the fluorescence patterns were recorded as described above.

(ix) Fractionation of Embryonic Extract: Embryonic homogenates were subjected to a fractionation procedure described in detail in Chapter 5-2, section (i). Depending on the size of the embryos to be studied, it was necessary to pool from four to sixteen embryos to obtain enough material for identification of the fluorophores. A typical analysis involved two age groups, one of embryos of H-H. stage 16-20 and the second of H-H. stage 28-30. A 1 ml aliquot of each fraction was diluted to a final volume of 3 ml with buffer "B" and examined fluorometrically. The relative fluorescence of each emission peak was then multiplied by the total volume of that fraction, in order to approximate the fluorophore content of each fraction. The intensity of fluorescence was further corrected against the acridine orange standard solution (0.01 mM in distilled water) with excitation at 370 nm and emission at 525 nm.

(x) Examination of the Instability of the 470 nm Emission Component: The following approaches were taken to examine the stability of the 470nm emission peak. (a) Short term storage conditions: The fluorescence spectra of the post-nuclear supernatants were compared from two embryos (H-H. stage 27), one frozen in dry ice-acetone and the other frozen in

liquid nitrogen. (b) Alkaline treatment: The post-nuclear supernatant from an embryo of state 28 was adjusted to pH 11 by addition of 0.1 N NaOH, then allowed to stand at room temperature for 30 minutes. The solution was then neutralized with 0.1 N HCl and brought to a final pH of 7.8. This solution was then examined by fluorometry and the pattern compared to the original one. (c) Photo-decomposition: The relative fluorescence of the 440 nm and 470nm emission peaks were determined from the post-nuclear supernatant of a stage 28 embryo homogenate. The supernatant was exposed to the xenon lamp of the fluorospectrophotometer set at a wavelength of 370nm, for 90 minutes. The relative fluorescence was again determined for the 440nm and 470nm emission peaks. (d) Prolonged storage conditions: The fluorescence spectra of the post-nuclear supernatant of stage 28 embryos were examined after storage at -20°C for approximately one year, and the results were compared with those obtained from the same sample one year earlier (first measured by Igarashi).

(xi) Fluorometric Analysis of Solutions of L-Tyrosine and L-Tryptophane: Stock solutions of 1 mg/ml of crystalline L-tyrosine and L-tryptophane were prepared in double distilled water. Each solution was kept on ice and the entire flask wrapped in aluminum foil, to prevent photo-decomposition. The desired dilutions (from 0.01 mM to 0.5 mM) were made with double distilled water. The spectral patterns were recorded over a range of dilutions and the relative fluorescence of each peak at each dilution was quantitated.

(xii) Fluorometric Analysis of Coenzyme Solutions: Stock solution (1mM) of riboflavin (RF), flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) were prepared in double distilled water. Each sample was then brought to a final concentration of 0.5 mM in buffer "B" pH 7.8.

The fluorescent characteristics of these substances were recorded and compared.

(xiii) Chromatographic Techniques: The following chromatographic procedures were used in attempts to purify the embryonic fluorophores. The effluent from the following columns was channeled through a quartz flow-through cell for the measurement of fluorescence. (a) DEAE-cellulose chromatography: A 25ml column of DEAE-cellulose was equilibrated with buffer "K" (Igarashi, 1969) at pH 7.0. A 5ml sample of the soluble fraction was obtained by fractionation of the homogenate of four embryos of stage 28 to 29 according to the procedure outlined in section (ix). This sample was diluted with buffer "K" to give a final concentration of 35 mM KCl. The effluent was monitored with the Baird-Atomic Fluorispec with excitation set at 370nm and emission set at 460nm. The sample was loaded and the column washed with 100 ml of buffer "K", followed by a KCl gradient from 0 to 500 mM KCl in buffer "K". The elution patterns were recorded on a YSI recorder (Model 81A, Yellow Springs Instruments Co., Yellow Springs, Ohio). (b) Phosphocellulose chromatography: A 25 ml column of phosphocellulose was equilibrated with buffer "K", at pH 7.0. The remaining procedures were the same as described for DEAE-cellulose chromatography. (c) Sephadex G-10 chromatography: A 25 ml sephadex G-10 column was equilibrated with buffer "K" at pH 7.0. A 3 ml sample of the soluble fraction obtained from embryos of H-H. stage 28 was prepared as described previously. The undiluted sample was applied to the column and the column was washed with buffer "K". The elution pattern was monitored fluorometrically, as previously described for ion exchange chromatography.

(xiv) Ethanol Precipitation Method: Each sample was brought to a final

concentration of 75 percent ethanol by the addition of 98 percent ethanol. The sample was then placed on ice for one hour to permit full precipitation. It was then centrifuged at 12000 x g. at 4°C for 10 minutes in a Beckman J-21 refrigerated centrifuge. The ethanolic supernatant was decanted and kept on ice in the dark for fluorometric analysis later. The insoluble pellet was suspended in buffer "B" at pH 7.8, then brought to pH 11 by 0.1 N NaOH. This sample was saponified at 37°C in the dark for 16 hours. The saponified sample was neutralized with 0.1 N HCl to pH 7.8 and examined fluorometrically along with the original ethanolic supernatant.

(xv) Chloroform Extraction Method: An equal volume of water-saturated chloroform was added to each sample. The mixture was then vortexed 5 minutes and spun at 2,000 x g at 4°C for 10 minutes in the J-21 refrigerated centrifuge. Three layers were formed: a top buffer layer, a middle denatured protein layer, and the bottom chloroform layer. The buffer layer was pipetted off first. The chloroform layer was then removed, evaporated to dryness, and the residue suspended in buffer "B". The middle, denatured layer could be resuspended in buffer in one of two ways: (1) dissolved in 9 M Urea, or (2) dried completely and suspended in buffer "B". The fluorescence spectra could then be recorded for each of the resulting fractions.

(xvi) Enzymatic Digestions: Stock solutions (10 mg/ml) of the following enzymes were prepared in double distilled water: α -chymotrypsin, pronase, and hyaluronidase. The precise amount of enzyme used in each digestion is described in Chapter 5 in the sections dealing with the enzymatic digestion. Each digestion followed the same procedure unless otherwise specified. After addition of the enzyme, the sample (in buffer

"B" at pH 7.8) was mixed, and incubated in the dark at 37°C for 16 hours. The digested sample was then centrifuged at 12,000 x g for 10 minutes at 4°C in a Beckman J-21 refrigerated centrifuge. The supernatant was then removed, and its pH was checked to make sure it had remained at pH 7.8. The sample was kept in the cold in the dark until treatment or analysis.

(xvii) TCA Precipitation Method: The volume of each sample subjected to trichloroacetic acid precipitation was first brought to the concentration of 5 percent TCA by addition of an equal volume of 10 percent TCA. This solution was mixed by vortexing, and placed on ice for one hour. The solution was then centrifuged at 12,000 x g at 4°C for 10 minutes in the J-21 refrigerated centrifuge. The acidic supernatant was decanted and brought to pH 7.8 by addition of 0.01 N NaOH. The acid precipitate was solubilized by addition of 0.5 ml of 0.01 N NaOH. This alkaline solution was neutralized with the addition of approximately 0.5 ml of 0.01 N HCl; then the solution was brought to pH 7.8 with the addition of 1 M Tris buffer (pH 7.8) to a final concentration of 0.1 M Tris. Both the TCA-soluble and the TCA-insoluble fractions were examined fluorometrically, and each emission peak was quantitated.

CHAPTER 3

GROWTH PARAMETERS FOR THE CHICK EMBRYO

3-1. Introduction:

In work described in Chapter 1, it was found that acceleration of embryonic development due to light exposure is detectable as early as in the first week of development (Isakson *et. al.*, 1970; Lauber, 1975). Preliminary studies, concerned with identification of substances possibly involved in this acceleration, revealed that there are changes in the fluorescence spectra between the third and sixth day of development (Lauber and Kato, Igarashi, unpublished data). In pursuing this line of studies, it was felt that the instability of fluorophores in the embryonic extract must be taken into consideration. During the preparation of embryonic extract in the future studies, it was decided to freeze each embryo as quickly as possible after removal from the egg, thus minimizing the decomposition of fluorophores. This made it necessary to devise a simple but accurate means of recording and identifying the developmental stage of individual embryos up to six days of development. Only then may it be possible to correlate the morphological events to fluorometric analyses which will be described in Chapter 4. This chapter is devoted entirely to establishing the relationship between H-H. stage (stages 16 to 30) and such growth parameters as body weight and dimensions of various parts of the chick embryo.

In hopes of finding a growth parameter which could be quickly measured and correlated with the developmental stage, the side view photographs were taken of individual embryos of H-H. stage 16 to 30 (approximately three to six days). We measured the brain region, which is disproportionally large and grows rapidly during this period (Romanoff, 1960; Garrigan, 1963). A strict correlation was found between the

cranial length and both body wet weight and H-H. stage.

It was also necessary to examine the effect of incubation conditions on the above correlation. Even minor deviations from the "optimal" incubation conditions (for review, see Landauer, 1961), in particular temperature, may have pronounced effects on normal growth and development (Romanoff, 1936 and 1938; Cruz, et.al., 1968). The difference in incubation conditions may offset the correlation between a growth parameter and the developmental stage. Upon examination, the correlation between cranial length, body wet weight and H-H. stage was found to be unaffected by the incubation temperatures ranging from 35.5 to 38.5°C.

Using the established correlation, each embryo could be washed, weighed, photographed, and frozen within two minutes after removal from the egg, thus minimizing the decomposition of possibly unstable chromophore. The permanent photographic record permitted us to estimate the developmental stage by a simple measurement on the photographic negative. The developmental stage of a particular embryo used in the fluorometric analysis can thus be determined at a later date. The procedure allows us to pinpoint the time in development when any changes in fluorescent pattern take place, as well as to compare these findings with past studies dealt with the same period of development.

3-2. Results:

(1) Correlation between H-H. Stage, Body Weight, and Cranial Length:

The first experiment was designed to investigate the relationship between the cranial length, wet weight and the H-H. stages from 16 to 30. The eggs were incubated under the conditions known to be optimal for normal development as reviewed by Landauer (1961). The equipment and incubation conditions utilized are described in detail in Chapter 2-3 (ii).

It was necessary to remove eggs for sampling frequently over the

seven days of incubation. It was thus desirable that the incubator utilized rewarm rapidly after being opened. A forced draft incubator was chosen since it regains an operating temperature within five minutes after the door has been opened. Thus the effect of temperature fluctuation was minimal. Also, because many other factors can lead to variation in developmental rate (for a review see Landauer, 1961), the decision on the developmental stage of the individual embryos was based on assessment of growth parameters and morphological characteristics, rather than on incubation time per se.

In an attempt to establish an easily measured growth parameter, eggs were sampled every 12 hours, from 72 hours to 164 hours of incubation time. After removal from the egg, each embryo was washed with saline, photographed, and weighed, as described in Chapter 2-3 (iii). The embryo was then placed in Bouin's fixative for determination of the H-H. stage later. It was found that within the period of development of interest (stage 16-30) the long axis of the cranial area could be measured quite accurately on the photographic negative (Fig. 1).

Figure 2 shows the logarithm of body weight plotted against the logarithm of cranial length for 15 embryos. Over the range of body weights from 10 mg to 450 mg, and the range of cranial lengths from 1.5 mm to 10 mm, the two parameters are significantly correlated ($r = 0.96$). In embryos larger than 450 mg, the rate of growth of the brain region slows and the correlation between these parameters is lost.

In order to examine the relationship between each growth parameter and the H-H. stage, the cranial length (Fig. 3A) and the body weight (Fig. 3B) are plotted semi-logarithmically against the corresponding stage of each embryo. There is a linear relationship between the logarithm of each growth parameter and the H-H. stage, although the correlation between

Fig. 1. Cranial length measurement. The outline from a photograph of a stage 25 embryo (approximately five days) is shown. The measurement for the cranial length (CL) was made from the middle of the midbrain to the tip of the forebrain. This dimension can be measured in embryos from stage 16 to stage 30: CL, cranial length; MB, midbrain; FB, forebrain; E, eye; WB, wing bud; LB, leg bud.

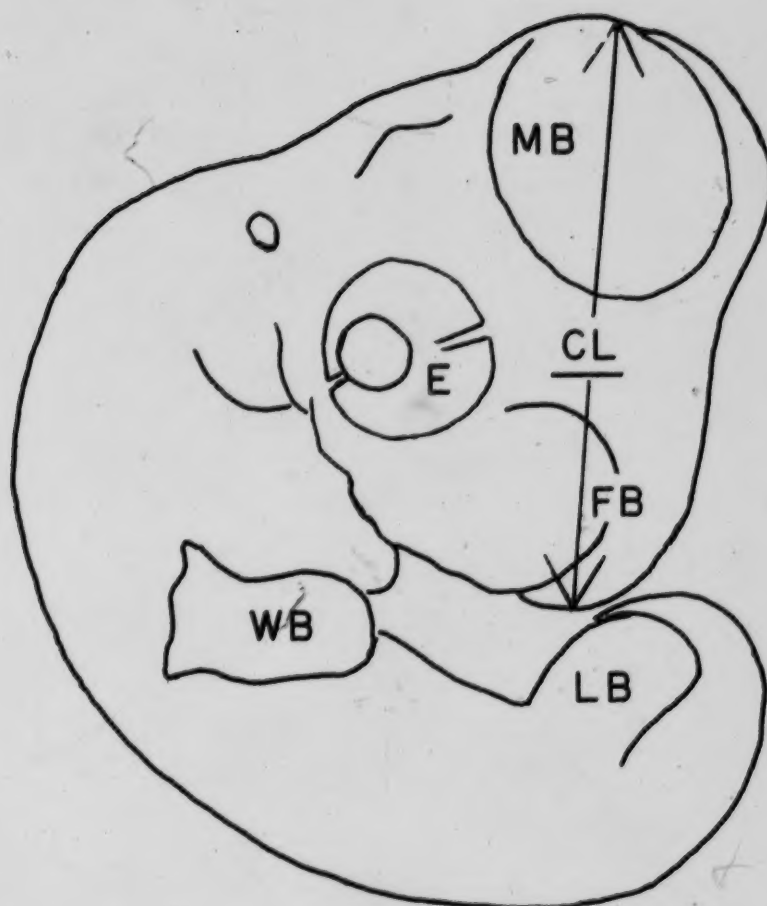


Fig. 2. Relationship between cranial length and body weight in chick embryos. Body weight is plotted against cranial length for 15 individual embryos on a log-log graph. The regression line has a slope of 2.15 and the correlation is highly significant ($r = 0.96$).

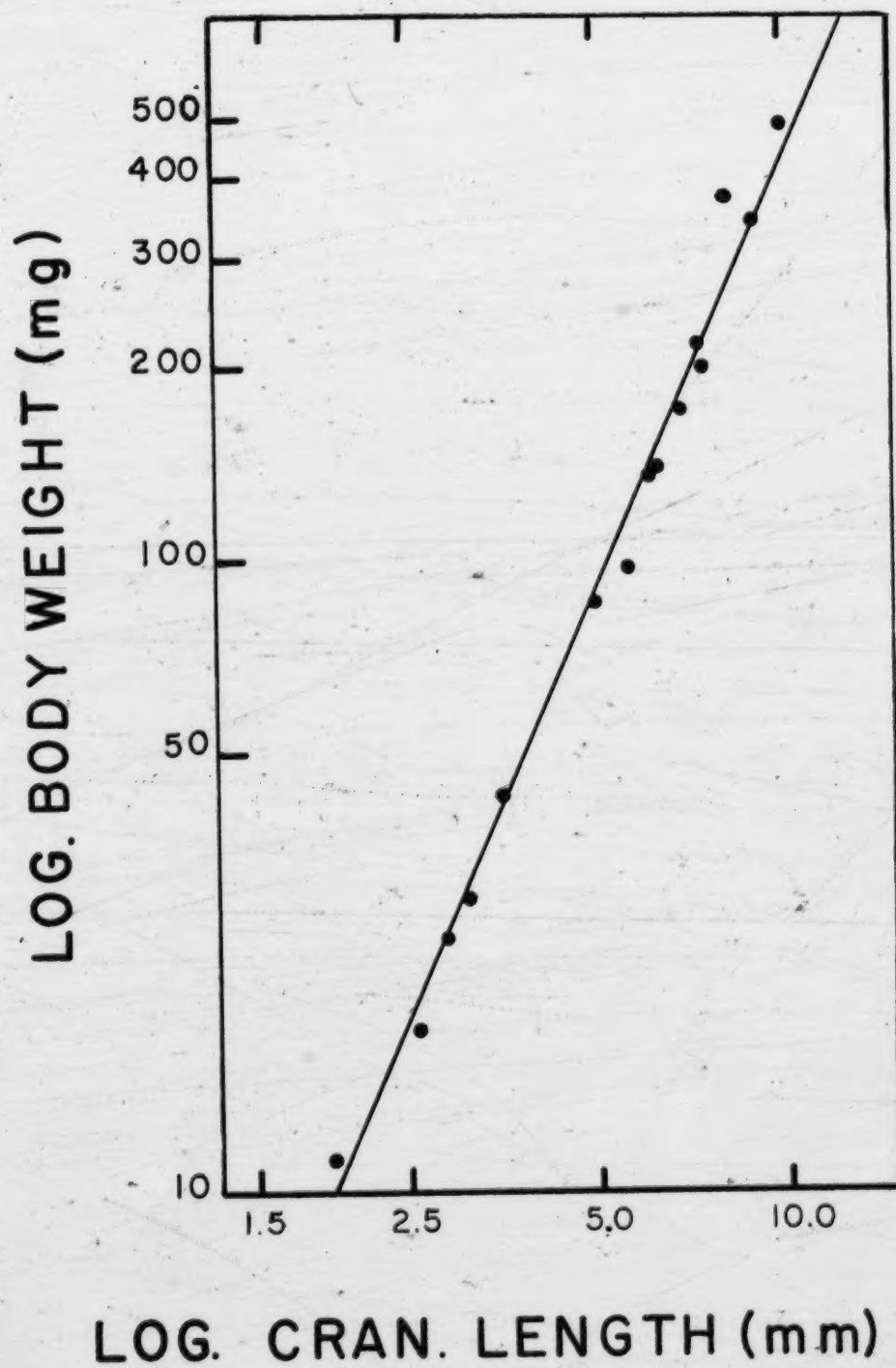


Fig. 3A. Relationship between the cranial length and the H-H. stage. The values of cranial length (▲) from 15 individual embryos are plotted against the H-H. stage as determined by careful observation of each embryo. The broken lines indicate the 80 percent confidence limits.

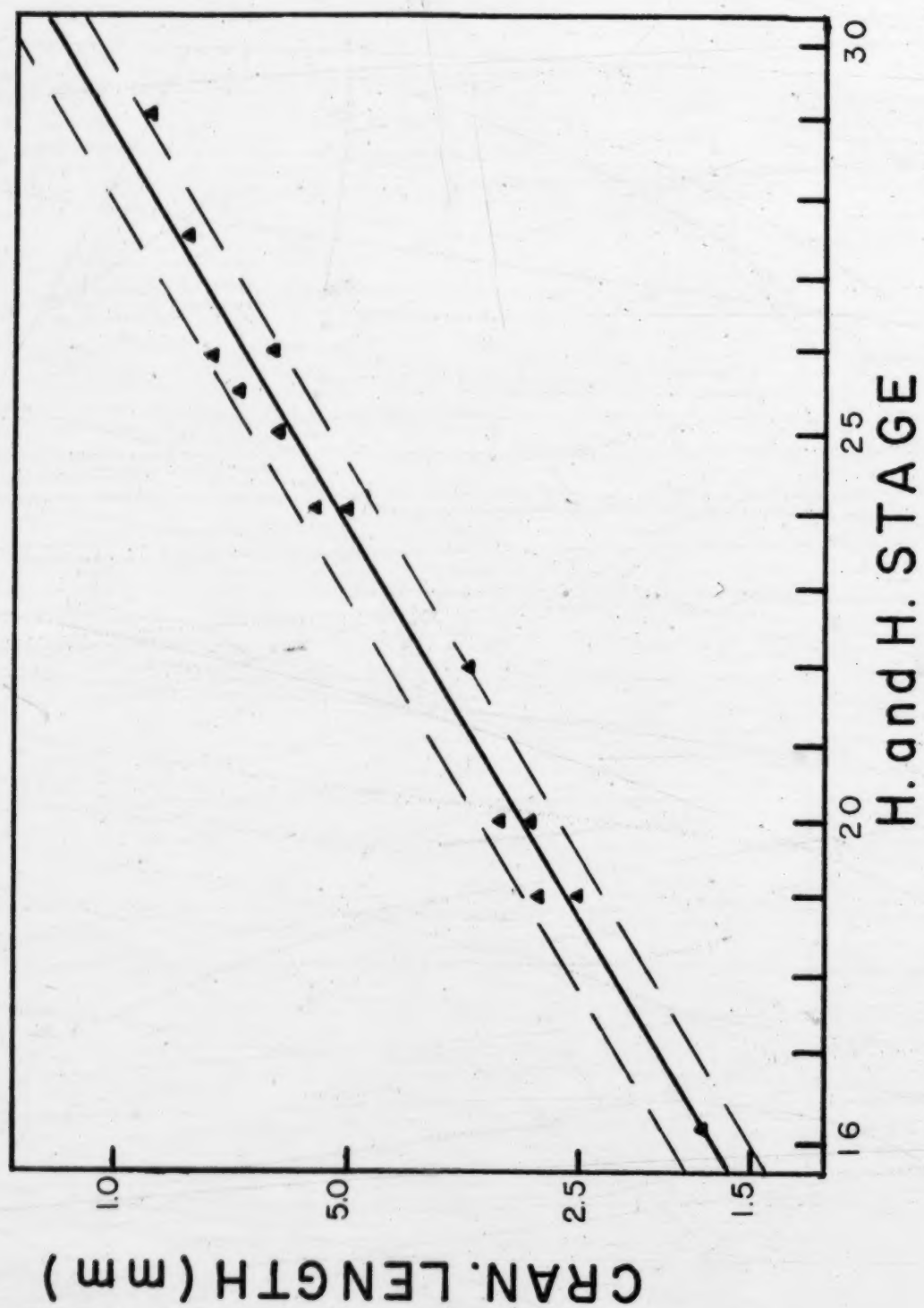
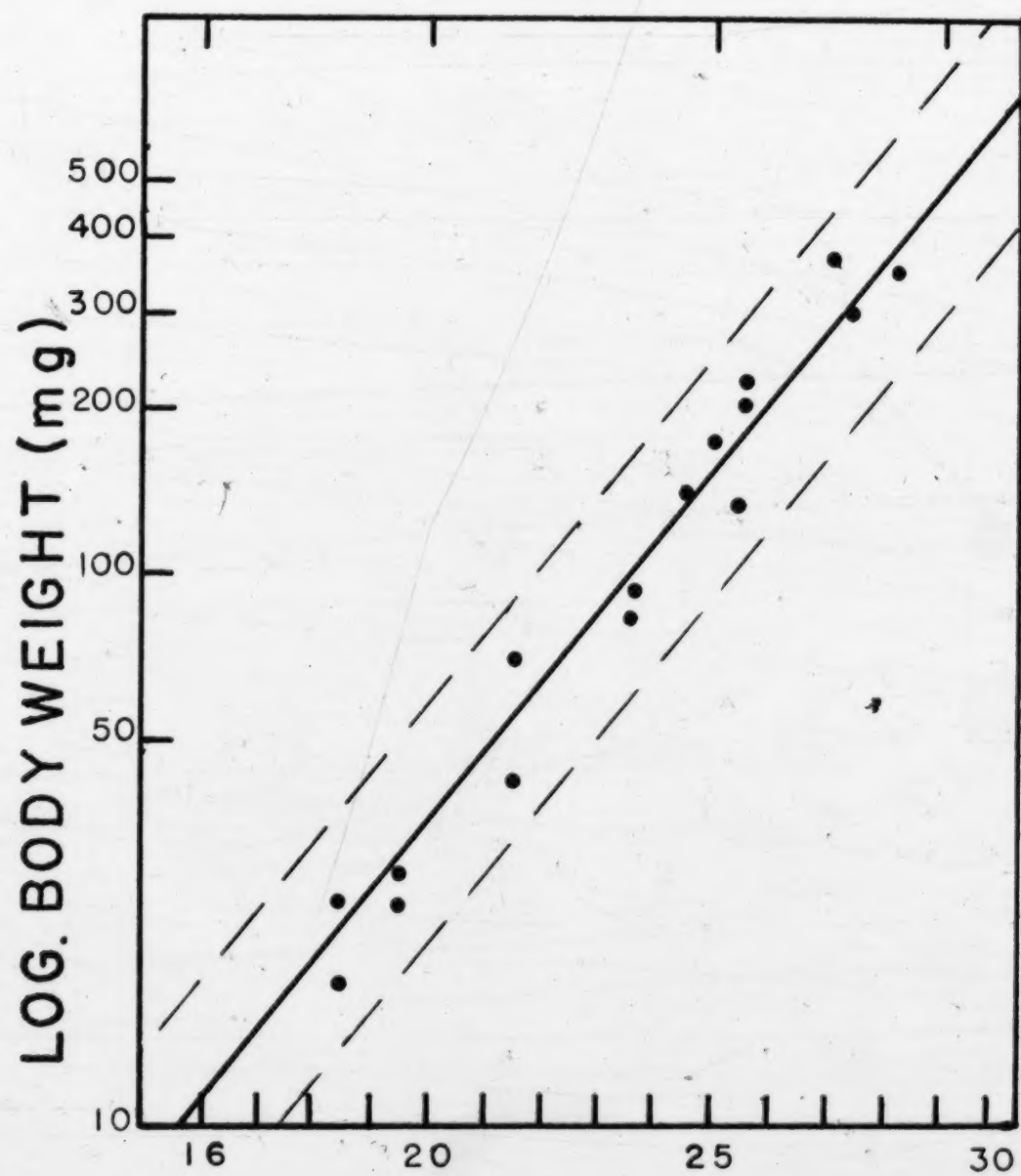


Fig. 3B. Relationship between body wet weight and the H-H. stage. The values of body weight (●) from 15 individual embryos are plotted semi-logarithmically against the H-H. stage as determined by careful observation of each embryo. The broken lines indicate 80 percent confidence limits.



H. and H. STAGE

the cranial length and the H-H. stage is lost beyond stage 29.

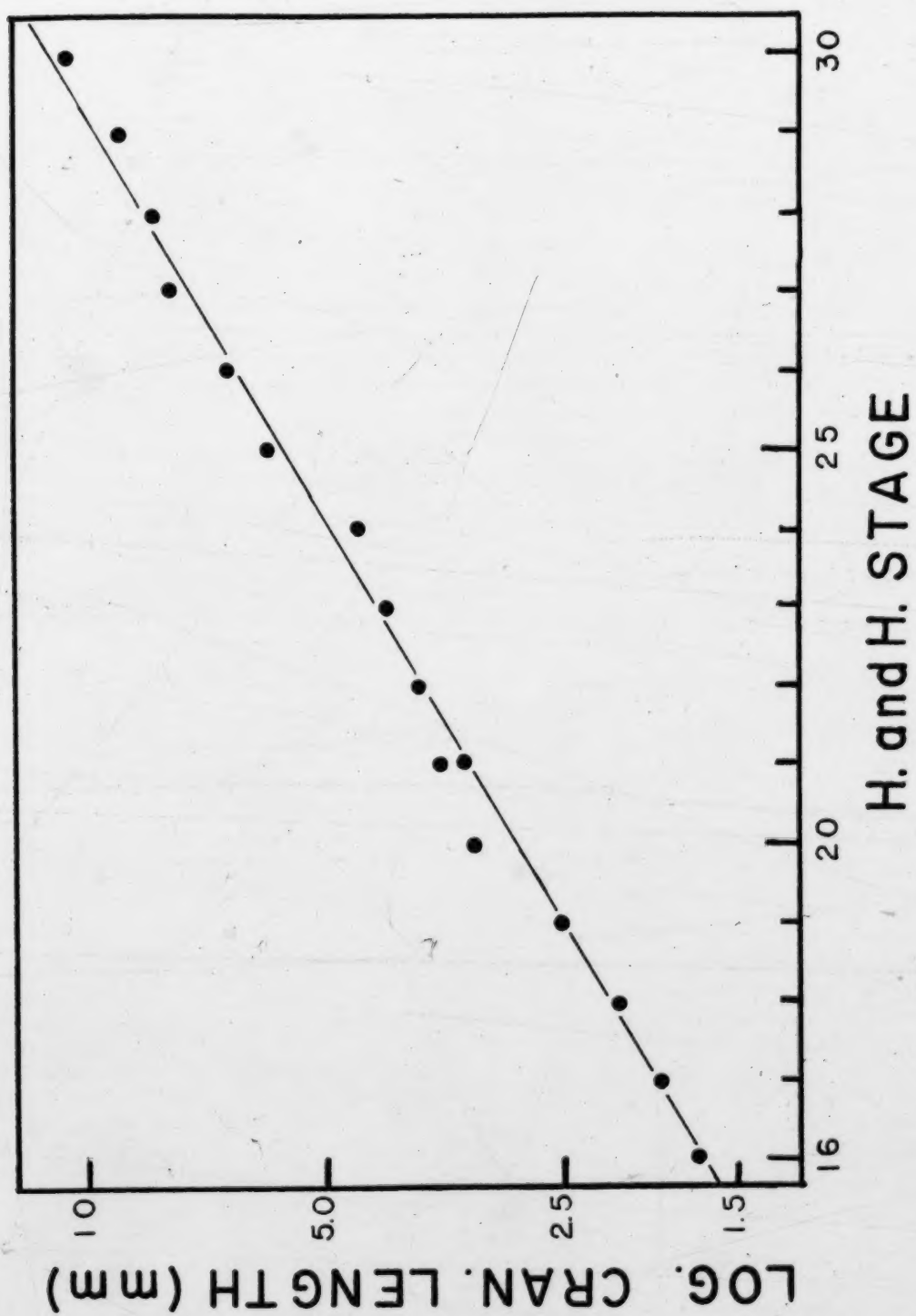
In order to provide an independent check on the correlation between H-H. stage and cranial length, the cranial length was measured from the photographs of the "typical specimens" for each stage, as presented in Hamilton's account of the H-H. staging system (1952). Figure 3C shows these values of cranial length, corrected to actual length ($1x$), plotted semi-logarithmically against the H-H. stage. The regression lines determined in Fig. 3A for our own embryos, and in Fig. 3C for the "typical specimens", have identical slopes. This supports the contention that our means of estimation of stage from cranial length is quite reliable, and its use provides a means of estimating the H-H. stage without the need for time-consuming morphological observations.

The usefulness of body weight as a means of estimation is limited. A considerable degree of error may be introduced into the weight values of the smaller embryos (stages 16-24), due to incomplete removal of the saline used to wash the embryos. However, this error is reduced beyond stage 24 as the body weight begins to increase logarithmically. By stages 29 and 30, where the cranial length estimate can no longer be used to assess H-H. stage accurately, the body weight values may be used to estimate stage.

(ii) Temperature Effect on the Correlation Between Three Growth Parameters:

It is well known that the incubation temperature has a direct effect on the rate of embryonic growth and on the differential growth of various organs. It has also been established that temperature extremes increase the incidence of mortality or embryo malformation (Romanoff, 1936; 1938; 1939). Therefore, we undertook a study to determine if small differences in incubation temperature could affect the correlation between the three growth parameters established in section (i).

Fig. 3C. Relationship between cranial length measured on Hamilton's (1952) example illustrations, and H-H. stage. The cranial lengths were measured from the photographs in Hamilton's text book of the embryos typical of each stage of development. These values are plotted semi-logarithmically against the corresponding stage. The regression line determined is identical to that in Fig. 3A.

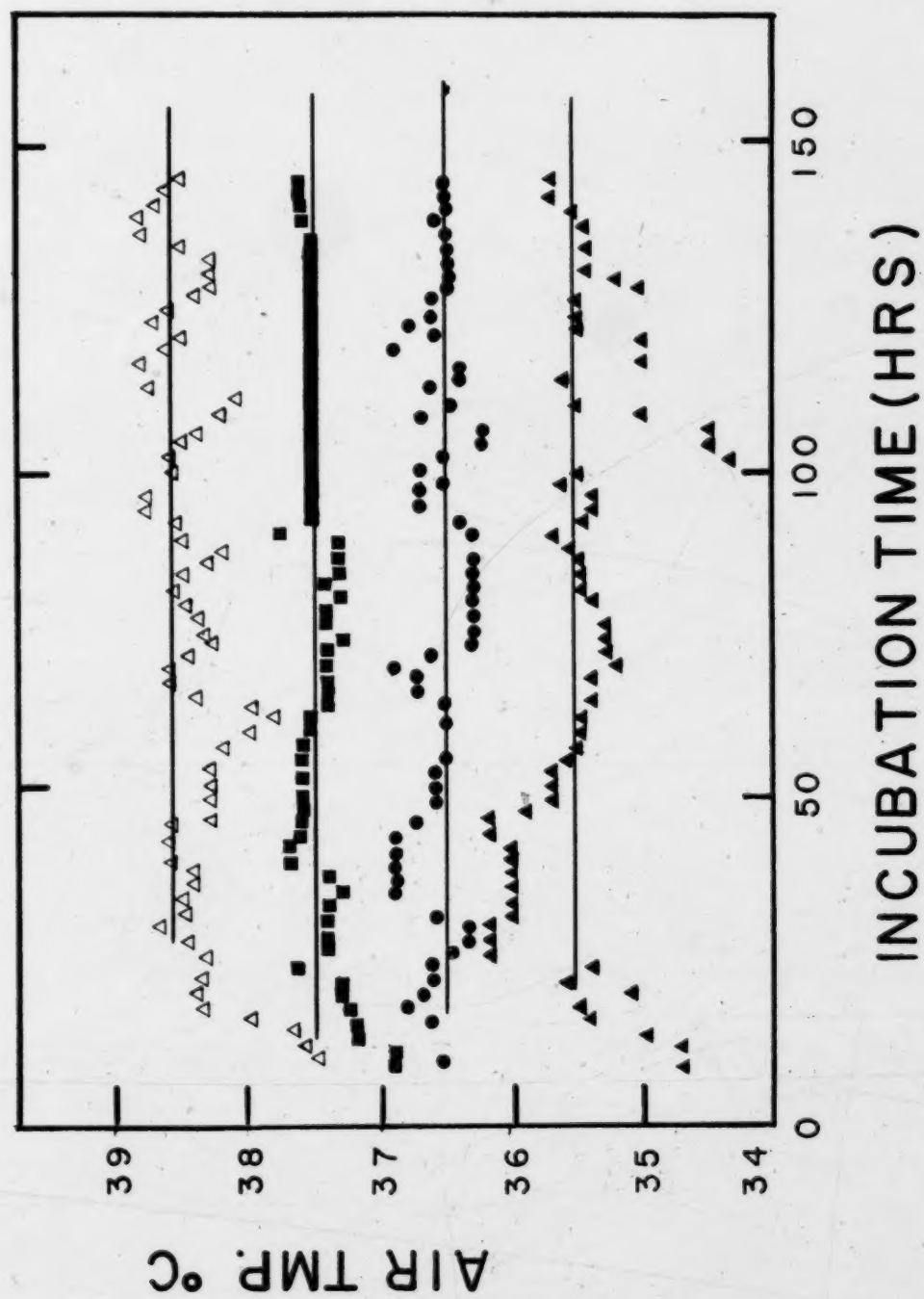


The water-jacketed incubator chosen was one in which the actual air temperature varied $\pm 0.15^{\circ}\text{C}$ around the selected temperature over a six-hour period. In each experiment the air cell temperature was monitored in four eggs, placed from the front to the back of the incubator, with Cu/Con P-38 thermocouples inserted into the air cells. The air temperature of the incubator was monitored as before, by a Rustrak thermistor air probe placed at egg level in the center of the incubator, and recorded on a Rustrak strip chart recorder. The relative humidity was maintained at 50 to 60 percent at all incubation temperatures, and an air flow of pre-warmed air was set at 12 liters/minute to insure proper ventilation. The source of the equipment utilized, the methods of recording temperature, and the precise incubation conditions are outlined in Chapter 2-3 (ii).

Four experiments were conducted, four dozen eggs in each, at incubation temperature settings of 35.5°C , 36.5°C , 37.5°C , and 38.5°C as described in Chapter 2-3 (ii). The recordings of incubator air temperature made during each experiment are presented in Fig. 4. The air cell temperature of the eggs in the center of the incubator followed the recorded air temperature. From the front to the back of the incubator there was an air temperature gradient of approximately 0.8°C at each of the incubation temperature settings noted above. Due to this gradient, the air cell temperatures of the eggs throughout the incubator fell within a range of $\pm 0.4^{\circ}\text{C}$ around the recorded air temperature. However, because the eggs were kept at the same position within the incubator for the duration of each experiment, this temperature gradient need not be considered a factor leading to variation between experiments.

The factor of incubation time was reintroduced in order to demonstrate the effects of temperature on growth rate and on developmental

Fig. 4. Recordings of the temperature in the incubator over six days at four temperature settings. A continuous air temperature recording was made with a Rustrak thermistor air probe. Each point represents the air temperature in the center of the incubator at egg level read at two-hour intervals. (The air cell temperatures of the eggs were spread around air temperature $\pm 0.4^{\circ}\text{C}$.) The fluctuation of air temperature at each setting is presented. ($\blacktriangle$) 35.5°C ; ($\bullet$) 36.5°C ; ($\blacksquare$) 37.5°C ; and ($\blacktriangle$) 38.5°C .



rate. During each of the four temperature experiments, twelve eggs (three from each of four egg trays) were removed daily, from the third to the sixth day of incubation. The embryos were then weighed, photographed, and frozen in liquid nitrogen. Before examining the differences in growth rate due to incubation temperature, it is necessary to establish that there was not excessive malformation or mortality at any of the temperatures utilized. Table I lists the percent of early death and the number of embryos normally developed at the end of six days of incubation at each incubation temperature. Embryos obviously deformed or weighing more than 2.5 times the S.E.M. of the mean body weight of that group were considered abnormal. This table shows that both the highest mortality, 11 percent, and the lowest normal development, 77 percent, occurred in the eggs incubated at 35.5°C. This result was expected in view of the known effect of deviation from the optimal incubation temperature (Romanoff, 1936, 1938; Cruz, *et. al.* (1968) .

Table II presents the mean values of cranial length and of body weight, in terms of percent variation from the values obtained under conditions defined as "optimal" for normal growth and development (review by Landauer, 1961). The values obtained at the incubation temperature of 37.5°C were considered to be 100 percent normal growth. Examining Table II, by the sixth day of incubation (144 hours) at 35.5°C, 2°C below the optimal temperature, mean body weight was 50 percent less than the optimal, and 40 percent greater at 38.5°C, 1°C above optimal. Also the mean cranial length at 35.5°C, 2°C below the optimal temperature, was 30 percent less than that at optimal, and was 10 to 20 percent greater at 38.5°C, 1°C above the optimal incubation temperature. These results confirm that temperature is a major factor influencing growth rate.

TABLE I. Number of embryos developing normally at each of four incubation temperature settings.
Embryos were assessed at the end of six days (144 hours) of incubation.

Temperature of incubation, °C ± 0.4°C	Fertile eggs set	Percent early dead	Percent normally developed	Total number normally developed
35.5	44	11%	77%	35
36.5	45	4%	90%	40
37.5	44	9%	84%	38
38.5	44	6%	87%	40

TABLE II. The comparison of several growth parameters at four different incubation temperatures. The values of the 37.5°C group were taken as 100% for each day and those in the other incubation temperature groups were expressed relative to this reference value.

Day (hours)	Temperature of incubation C	Percentage of normal development Body Weight	Cranial Length
3 days (76 hours)	35.5	55%	75.2%
	36.5	71.5%	85%
	37.5	100%	100%
	38.5	126%	118%
4 days (96 hours)	35.5	56%	73.2%
	36.5	76.7%	77%
	37.5	100%	100%
	38.5	163%	117%
5 days (120 hours)	35.5	47%	68%
	36.5	63%	78.7%
	37.5	100%	100%
	38.5	136%	108%
6 days (144 hours)	35.5	47%	76%
	36.5	60%	84%
	37.5	100%	100%
	38.5	138%	103%

To further demonstrate the differential growth rate at various temperatures, the mean body weight (Fig. 5) and the mean cranial length (Fig. 6) from each day and temperature group (listed in Table III) are plotted against time of incubation. The Duncan's multiple-range test (Duncan, 1955) was performed,utilizing the mean values of body weight and cranial length. This test takes into account the total number of groups and the numbers in each group, compares the group means, and indicates which means are significantly different. The results of this test are illustrated as a series of vertical lines on the right hand column of Fig. 5 and Fig. 6. When the vertical lines do not overlap, the mean values are different at the 95 percent level of significance. The large differences in both growth parameters,seen between the temperature groups at day six, verify the effect of incubation temperature on growth rate as measured by these chosen parameters. Therefore, before these parameters are utilized to estimate the H-H. stage, it is necessary to determine if the correlations previously established for the relationships between the chosen parameters are altered by variation in incubation temperature.

Figure 7 demonstrates the relationship between body weight and cranial length,from embryos grown at various incubation temperatures. The mean values of body weight are plotted against the mean values of cranial length for each day and temperature group. All the values fall on the single regression line with a slope of 2.05, similar to the slope of 2.15 obtained in Fig.2 in which these parameters are compared from embryos grown at optimal temperature. These results indicate that the correlation between the two growth parameters is unaltered over the incubation temperature range examined.

In order to examine the effect of temperature on the correlation

Fig. 5. Incubation temperature effect on body wet weight in three-to-six-day embryos. After removal of extra-embryonic membranes, including the allantois, the embryo was washed in saline, then placed in a small weighing pan, excess saline was drawn off with a slip of filter paper, and the wet weight recorded. Each point represents the mean body wet weight plotted against incubation time in hours, and the numbers of embryos in each group are given in Table III. The values were subjected to the Duncan's multiple-range test, and the results are listed as a series of vertical lines on the right hand column of the figure. When the vertical lines do not overlap, the mean values are different at the 95 percent level of significance. Temperature settings were:

(A) 35.5°C; (B) 36.5°C; (C) 37.5°C; and (D) 38.5°C.

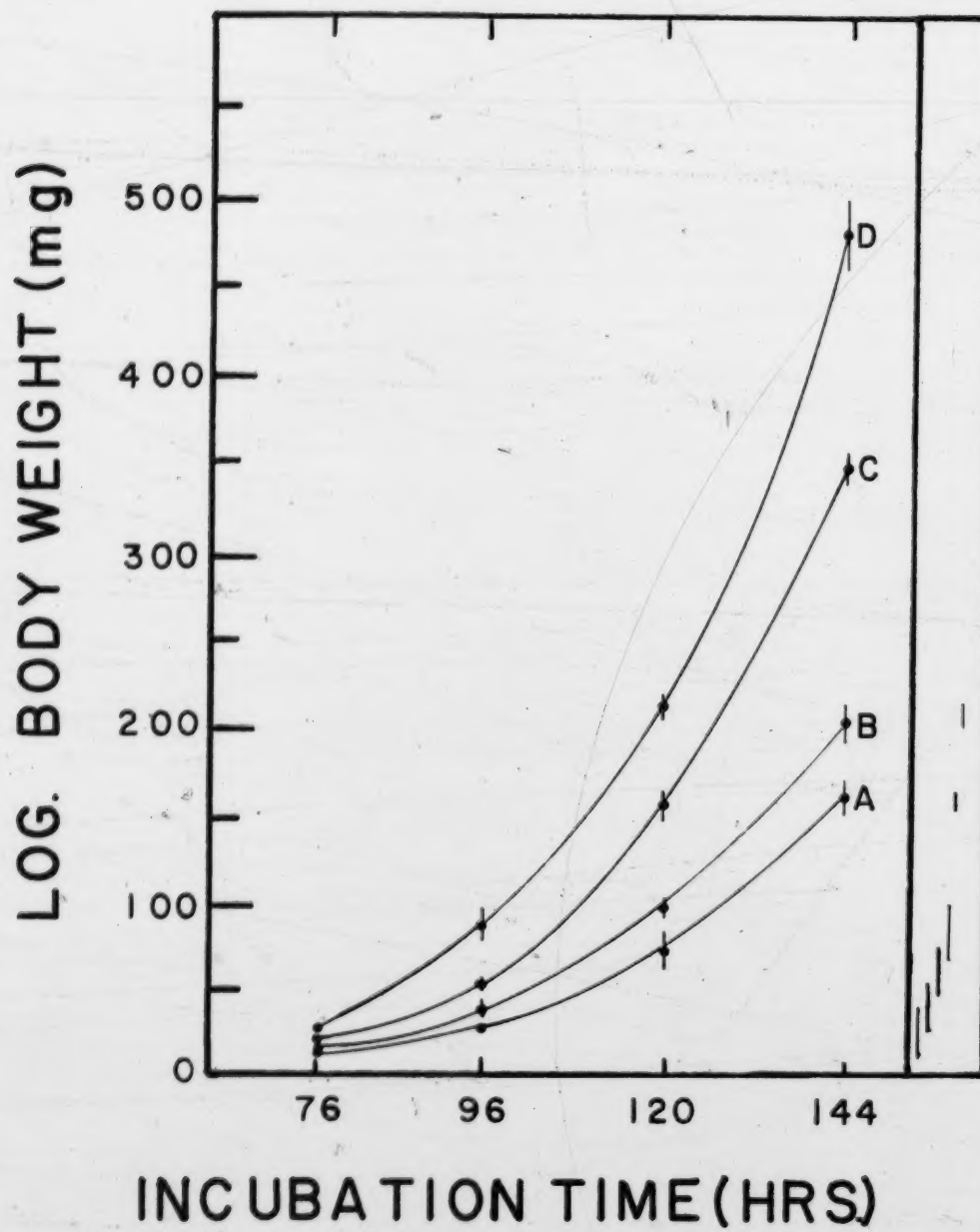


Fig. 6. Incubation temperature effect on cranial length in three-to-six-day embryos. The cranial length was measured on photographic c^cc negatives. Each point represents the mean cranial length plotted against incubation time in hours, and the numbers of embryos in each group are given in Table III. The values were subjected to the Duncan's multiple-range test, and the results are listed as a series of vertical lines on the right hand column of the figure. When the vertical lines do not overlap, the mean values are different at the 95 percent level of significance. Temperature settings were: (A) 35.5°C; (B) 36.5°C; (C) 37.5°C; and (D) 38.5°C.

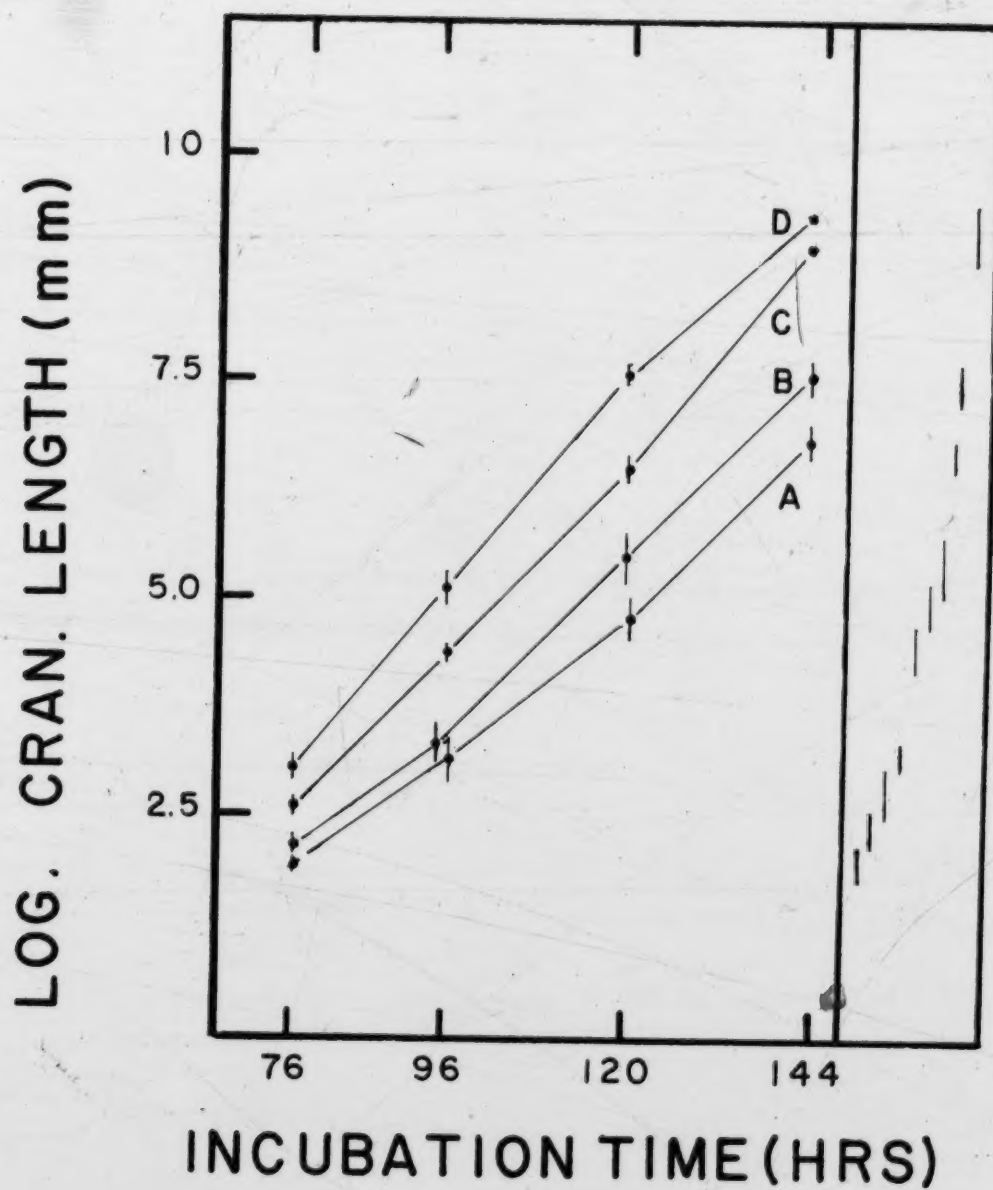
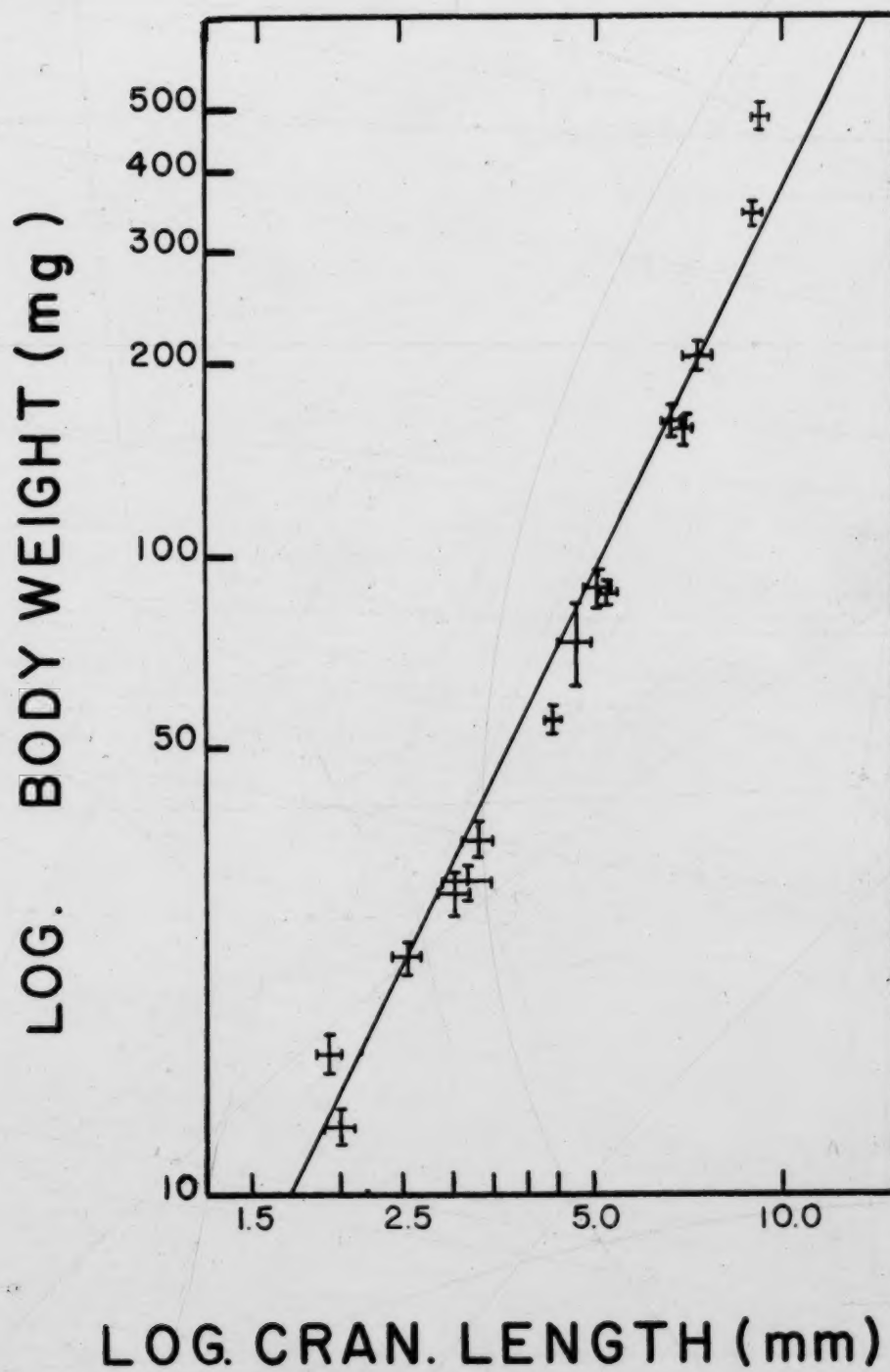


TABLE III. Statistical information on growth parameters from the four temperature experiments.

Day (hour)	Temperature °C	Body Wet Weight		Cranial Length	
		No. in Sample	mean mg	No. in Sample	mean mm
3 days (76 hours)	35.5	9	13.0	8	1.95
	36.5	10	16.8	10	2.20
	37.5	10	23.5	10	2.57
	38.5	11	29.6	11	3.05
4 days (96 hours)	35.5	8	30.63	8	3.17
	36.5	10	42.2	9	3.34
	37.5	9	55.0	9	4.34
	38.5	10	89.8	10	5.09
5 days (120 hours)	35.5	9	73.8	9	4.71
	36.5	11	99.0	11	5.50
	37.5	10	157.0	10	6.95
	38.5	9	212.9	9	7.52
6 days (144 hours)	35.5	9	163.2	9	6.80
	36.5	10	207.6	10	7.50
	37.5	9	347.6	8	8.95
	38.5	10	480.5	10	9.23

S.D.M: standard deviation of mean.

Fig. 7. Temperature effect on the body weight-cranial length relationship. The mean body weight and cranial length values from four temperature settings, along with the S.E.M. for each group at each temperature, are shown on the log-log plot. The regression line, with a slope of 2.05 and $r=0.92$, was determined utilizing the individual body weight and cranial length values from all the temperature experiments.



between these two parameters and the H-H. stage, an assignment of the H-H. stage of each embryo was made and recorded as the embryo was being photographed. Figure 8 shows the cranial length and Fig. 9 shows the body weight plotted semi-logarithmically against H-H. stage. The regression lines and the 80 percent confidence limits were determined and superimposed on each graph. Comparing the regression lines here with those determined in Fig. 3A and Fig. 3B respectively, the slopes are not significantly different at the 80 percent level of significance. The figures suggest that the relationship between the two growth parameters and the H-H. stage is not changed by variation in incubation temperature.

Finally, having established that overall growth rate is affected by incubation temperature, we attempted to determine whether the rate of development, in terms of H-H. stage attained, is affected to a similar degree. The mean values of cranial length listed in Table III were applied to the standard curve in Fig. 3A and the H-H. stage extrapolated and plotted against incubation time in Fig. 10. The resulting curves indicate differences in the rate of development between incubation temperature. On each day between three and six days of incubation the group of incubation temperature of 38.5°C , 1°C above optimal, is four to five H-H. stages ahead of the group at 35.5°C , 2°C below optimal temperature.

The above results indicate that although growth rate and developmental rate are influenced by incubation temperature, over the time period investigated, the relationships between the cranial length, body weight, and the H-H. stage are unaffected. Therefore, the selected parameters may be utilized to estimate the H-H. stage. Also, since the cranial length increases rapidly during the first portion of the developmental period studied, it presents a more precise parameter, with which to estimate H-H. stage during this period, than does the parameter of body

Fig. 8. Temperature effect on the relationship between cranial length and the H-H. stage. Individual cranial length values from four temperature settings are plotted semi-logarithmically against the H-H. stage. Each point represents a single embryo, whose H-H. stage was assigned during a brief observation while the embryo was being photographed. The regression line (solid) and the 80 percent confidence limits (broken lines) are presented. The temperature settings were: (▲) 35.5°C; (●) 36.5°C; (■) 37.5°C; and (△) 38.5°C.

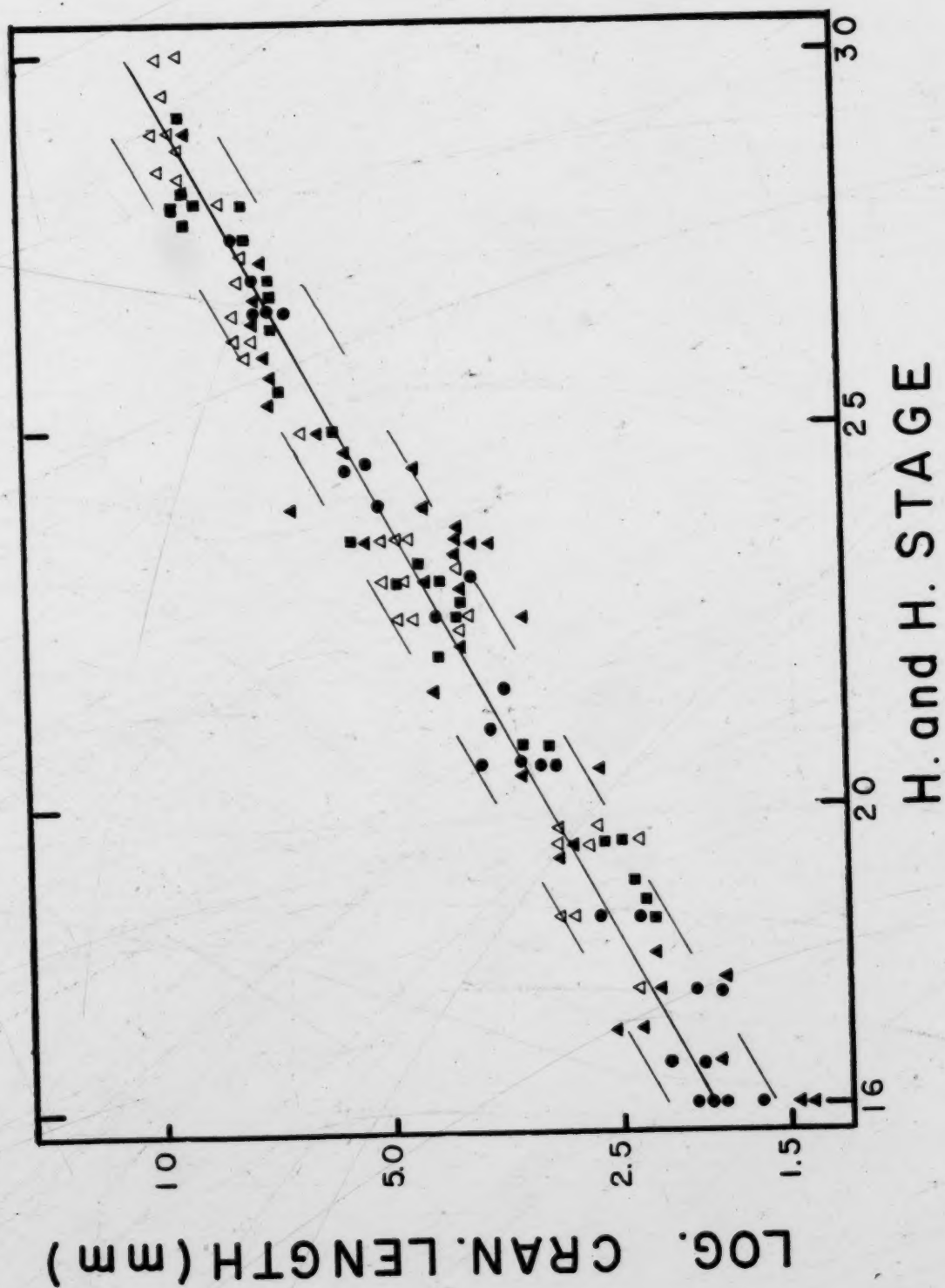
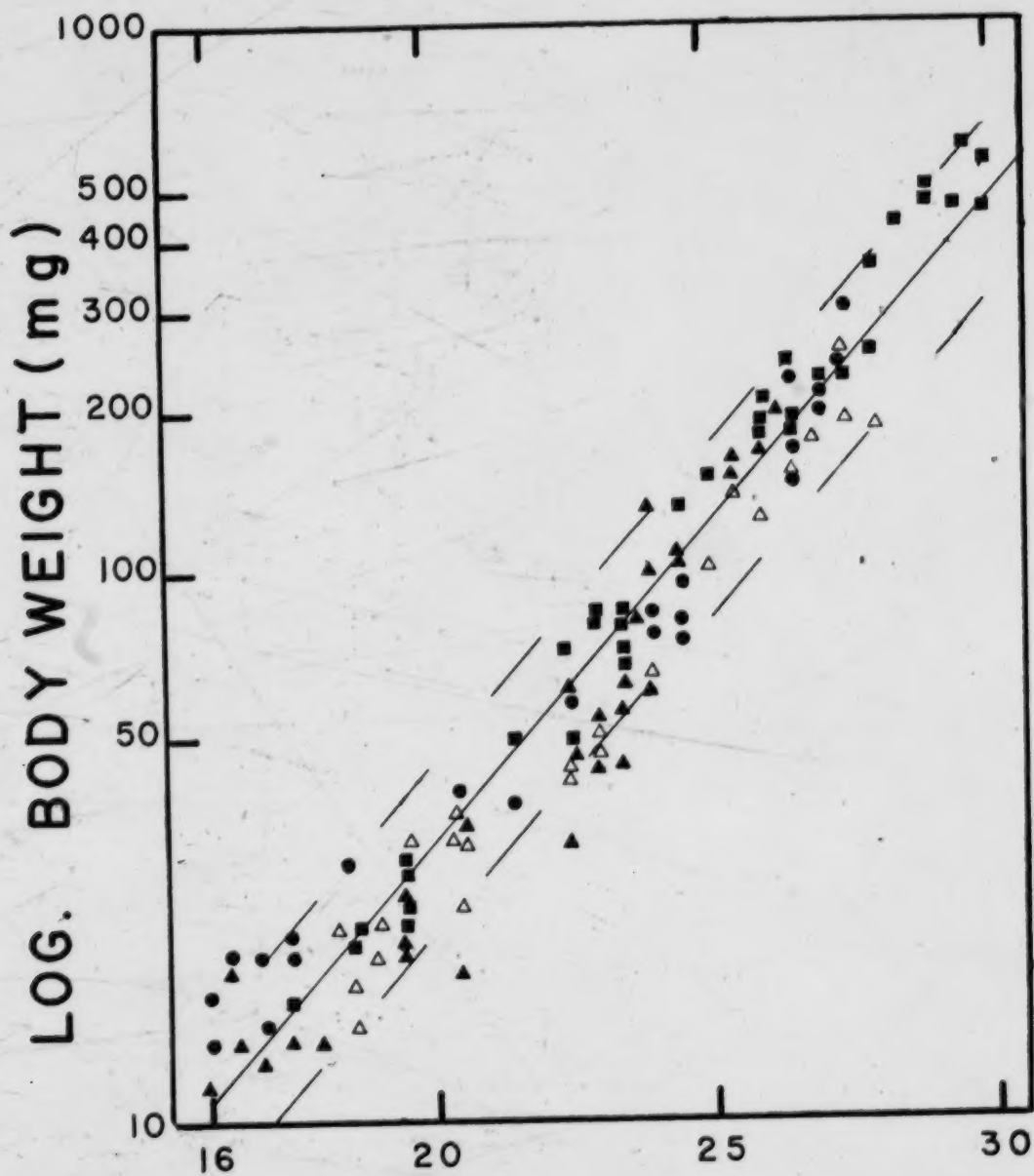
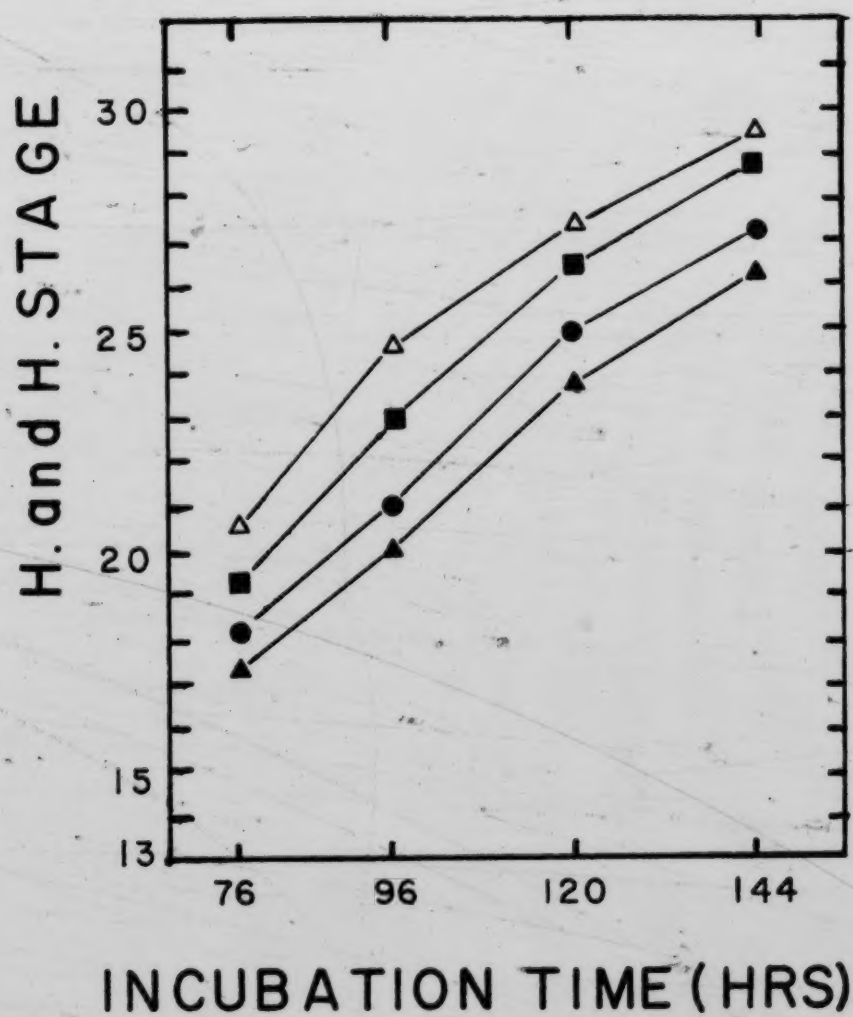


Fig. 9. Temperature effect on the relationship between the body wet weight and the H-H. stage. Individual body wet weight values from four temperature settings are plotted semi-logarithmically against the H-H. stage. Each point represents a single embryo, whose H-H. stage was assigned during a brief observation as the embryo was being photographed. The temperature settings were: (▲) 35.5°C; (●) 36.5°C; (■) 37.5°C; and (△) 38.5°C.



H. and H. STAGE

Fig. 10. Temperature effect on advancement of H-H. stage. Here stage has been estimated by applying the values of mean cranial length listed in Table III to the H-H. staging curve Figure 3A and extrapolating to the H-H. stage. The estimated H-H. stages are plotted against incubation period for each temperature group: ($\blacktriangle$) 35.5°C; ($\blacksquare$) 36.5°C; ($\bullet$) 37.5°C; and ($\triangle$) 38.5°C.



weight.

3-3. Discussion and Summary:

When investigating biochemical processes occurring during embryogenesis, many workers have found it necessary to pool embryos, particularly at the early stages of development, in order to obtain sufficient material for microchemical analyses. However, as Seltzer (1975) pointed out, the technique of fluorometric analysis is very sensitive and permits a minute quantity of embryonic material to be used. There is a possibility of qualitative, and even semi-quantitative, determination of certain fluorescent compounds in individual embryos as small as 10 mg of body weight. It therefore becomes important to know the precise developmental state of each of the individual embryos examined.

As noted in the introduction, a problem was encountered in the utilization of the conventional method of determining the developmental stage of the individual embryos for biochemical analyses. The components to be analyzed fluorometrically were possibly subjected to decomposition. A rapid, yet accurate, means of estimation of the developmental stage was therefore necessary.

In attempts to establish a method of quickly estimating the H-H. stage of embryos, it was hoped that a growth parameter could be measured from photographs of embryos and correlated with the developmental stage. We attempted to assess various growth parameters of the chick embryo, such as length-width ratios of the limb buds and the degree of eye pigmentation, both used in past studies (Hamburger and Hamilton, 1951) in correlation with the H-H. stage. However, the leg and wing buds were difficult to measure on an unfixed embryo, and the degree of eye pigmentation could not be judged accurately from a photograph. This necessitated the search for a more readily analyzed parameter.

We noted, as have other workers (Gerrigan, 1963; Romanoff, 1960), that the brain region of the chick embryo is quite prominent and increases rapidly during the period of development investigated here. When utilized as a growth parameter, brain length is easily and accurately measured on a photographic negative. This parameter, referred to in this study as cranial length, was found to be highly correlated both with body weight and H-H. stage, under incubation conditions considered optimal for normal development. Body weight was also found to be correlated with the H-H. stage. Based on these observations, standard curves were prepared, from which the H-H. stage of development may be extrapolated from each growth parameter, cranial length, and body weight. However, the use of the cranial length measurement in estimating the H-H. stage is simpler and more accurate than the use of body weight, over the developmental period of interest in this investigation.

As noted in the introduction, the selected growth parameters could not yet be utilized to estimate developmental stage without an assurance that the correlations between these growth parameters and the H-H. stage are not affected by minor variations from the incubation conditions considered as optimal. Because temperature is the incubation condition which exerts the most pronounced effect on normal growth and development (Romanoff, 1936; 1938), we undertook a study in which all other factors were held constant and the incubation temperature was varied.

The differential growth of embryos was examined from the third to the sixth day of incubation, over a range of incubation temperatures from 35.5°C, 2°C below the temperature defined as "optimal", to 38.5°C, 1°C above. By the sixth day in incubation, large differences were seen between temperature groups. At the higher incubation temperatures, the

mean cranial lengths and body weight were much greater than in the lower temperature groups. Mortality and malformation rates were comparable at each temperature. The differences in the body weight values are similar to the values presented by Romanoff (1960, p. 1145), obtained in several studies conducted over the same range of incubation temperatures used here. The results demonstrate that minor deviation from "optimal" incubation conditions has a major effect on the rate of growth.

It was difficult to quantitate a precise rate of development, since the developmental state is defined in the arbitrary units of H-H. stage. However, we were able to demonstrate differences in the developmental stages attained at different incubation temperatures. During the incubation period of interest here, the group of embryos raised at "optimal" incubation temperature (37.5°C) were always two to three H-H. stages ahead of the embryos incubated at 2°C below "optimal", and were one to two stages behind embryos grown at 1°C above "optimal" incubation temperature.

Therefore, we have been able to demonstrate that growth rate, and indirectly developmental rate, is increased at above-optimal incubation temperatures, whereas the reverse is found at sub-optimal temperatures. However, incubation temperature has no effect on the relationship between the two growth parameters, body weight and cranial length, nor does it alter significantly the correlation between those growth parameters and the H-H. stages. This confirms the accuracy of the use of the cranial length measurement as a means for assessment of the H-H. stage of individual embryos.

The use of this staging method allows embryos to be frozen shortly after removal from the egg, thereby minimizing the possibility of decomposition of unstable biochemical entities. By weighing and

photographing each embryo before freezing, a record of size and visual record of developmental stage can be maintained. The H-H. stage may be estimated from the cranial length at a later time and this information related to the results of the analysis of each embryo. Because minor differences in incubation temperature do not alter the correlation established between cranial length and stage, the chronological variation and the effects of slight deviations from optimal incubation conditions may be reassessed and normalized according to this staging method. This permits the comparison of the results of our biochemical studies (Chapter 4 and 5) with the results of other investigations concerned with the first week of embryonic development.

In conclusion, the experiments presented in this chapter were undertaken in order to define the developmental stage of individual embryos in terms of easily measured growth parameters. We were able to establish a means of analysis which (1) provides a simple means by which the developmental stage of individual embryos could be assessed from the growth parameters of body weight and cranial length; the latter measurement proved to be the simplest and most accurate means of estimating the H-H. stage; (2) permits the comparison of our observations with the results of other workers, without the need to consider minor differences in incubation conditions; and (3) allows the embryos to be frozen quickly, thus forestalling degradation of embryonic compounds to be later analyzed by fluorometric or other biochemical techniques.

CHAPTER 4

FLUOROMETRIC STUDY OF EMBRYONIC DEVELOPMENT

4-1. Introduction:

The second phase of this thesis deals with the fluorometric analyses of chick embryos during early development. As noted in Chapter 1, a preliminary investigation of the fluorescence spectra of chick embryos of three to six days of development had been conducted by Kato and Lauber (unpublished data). The embryos of certain body weight were pooled, dissolved in 75% sulfuric acid, and subjected to fluorospectrophotometric analysis. An increase in fluorescence intensity was detected as well as a shift in the emission peak as development progresses. However, the fact that the embryos were dissolved in sulfuric acid might have induced fluorescence by the formation of derivatives of certain compounds, e.g., steroids (Kalant, 1958; Albers, 1955). Therefore Igarashi (unpublished data) followed the early study by examining the fluorescence spectra of extracts from embryos of similar developmental age as used in the earlier study, but prepared by homogenizing the embryos in a buffered solution. The results confirm that there is indeed a spectral shift as development proceeds. He also found that chromophore(s) responsible for the spectral shift is localized in the post-mitochondrial supernatant. Thus, further study of chick embryogenesis by means of fluorospectrophotometry is warranted.

In the previous chapter, we established a precise staging method for chick embryos of three to six days of development. This method allows us to identify the stage of individual embryos and to analyze fluorescence spectra characteristics of any given embryo, instead of utilizing pooled embryos. The rationale behind the above approach is to pinpoint the exact stage at which spectral change, if any, takes place

during the early chick embryogenesis. Establishing the precise correlation between spectral change and the developmental stage, we may then advance our study towards characterization of chromophore or metabolic changes unique to a certain stage of morphogenesis and differentiation.

In pursuing the above line of study, we had to introduce several changes in the experimental procedures employed in the earlier studies by Kato and Lauber and by Igarashi. The key was to prepare embryonic extract with a buffering solution containing anti-oxidant (BME), which is necessary for maintaining at least some enzymic activities of the extract. We then monitored the fluorescence spectra of individual embryos, instead of pooled embryos, at the maximum sensitivity of a spectrophotometer, so as to detect the change in relative fluorescence as well as fluorescence maxima.

The analyses revealed three major emission peaks: 350nm, 520-525nm, and 430-470nm. Of these three peaks, the first two show gradual increase in relative fluorescence as development proceeds, but without significant shift in emission maxima. However, the third component showed not only increase in intensity but also a dramatic shift in emission maximum. The observed shift in emission maxima was found to be stage specific. The midpoint of the shift is at H-H. stage 23, and is unaffected by incubation temperature. The implications of this finding will be discussed later in this chapter and in Chapter 5.

4-2. Results:

(i) Fluorescence Spectra Characteristic of Early Embryonic Development:

The post-nuclear supernatants from homogenates of individual embryos of known stage were prepared by the method described in Chapter 2-3 (v), and subjected to fluorometric analyses as described in Chapter 2-3 (vi). A typical result obtained from an embryo of H-H. stage 16 is

illustrated in Fig. 11. It is apparent that there are three emission peaks: one in the UV region, 350nm (peak 1 in Fig. 11), and two in the visible region, 430nm and 520nm (peak 2 and 3). From the above data, it is clear that the chick embryo at stage 16 contains three dominant fluorescent compounds, although there must be innumerable fluorophores within cells but at concentration levels undetectable even at the maximum sensitivity of the fluorospectrophotometer.

It was then important to find whether the fluorescence spectrum at stage 16 remains essentially the same as development proceeds to stage 30, or if spectral changes take place. Therefore, we studied the fluorescence spectra of embryos from stage 19 to 30 (three to six days) under conditions identical to those for the embryo in Fig. 11. The results are illustrated for the UV region in Fig. 12(A-D), and for the visible region in Fig. 13(A-D). Comparing the spectra obtained over the four days of development, it is obvious that two of the three peaks originally seen at stage 16 are altered very little, aside from large increases in fluorescence intensity, in the stage 30 embryo. The UV peak, accompanied by a 10-fold increase in fluorescence intensity, undergoes a spectral shift from an excitation peak at 290nm and emission peak at 348nm in the stage 19 (three-day) embryo, to a 295nm excitation and 349nm emission peak in the stage 30 (six-day) embryo. In the visible range, the 520nm emission peak present in the stage 16 embryo shifts slightly to a peak of 525nm in the stage 30 embryo, with an accompanying 20-fold increase in intensity. The excitation peaks become clearer as the amount of this substance increases, reducing interference from the other regions. The spectrum changes from a broad peak (350nm to 470nm) at stage 16, to a well-defined double excitation peak by stage 30. The shift to the 345nm excitation may be the result of secondary fluorescence, induced by a

Fig. 11. The three regions of fluorescence emission characteristically found in a stage 16 embryo. This figure presents the emission pattern observed in the post-nuclear supernatant of an H-H. stage 16 embryo prepared as described in the text. There are three regions of emission which dominate the fluorescence pattern: the first (1) region is represented by an emission peak at 348nm; the second (2), by an emission peak at 430nm; and the third (3), by an emission peak at 520 nm.

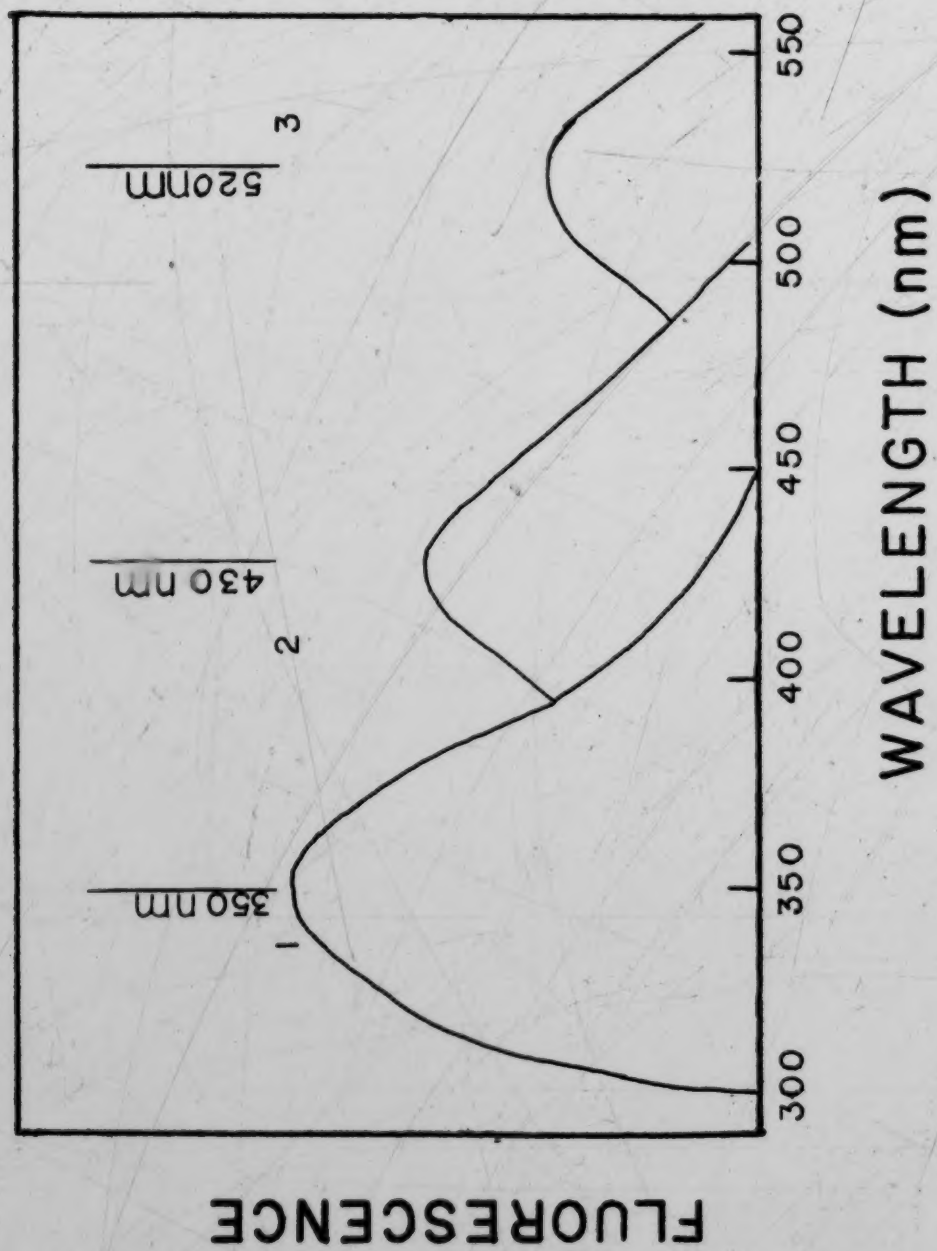


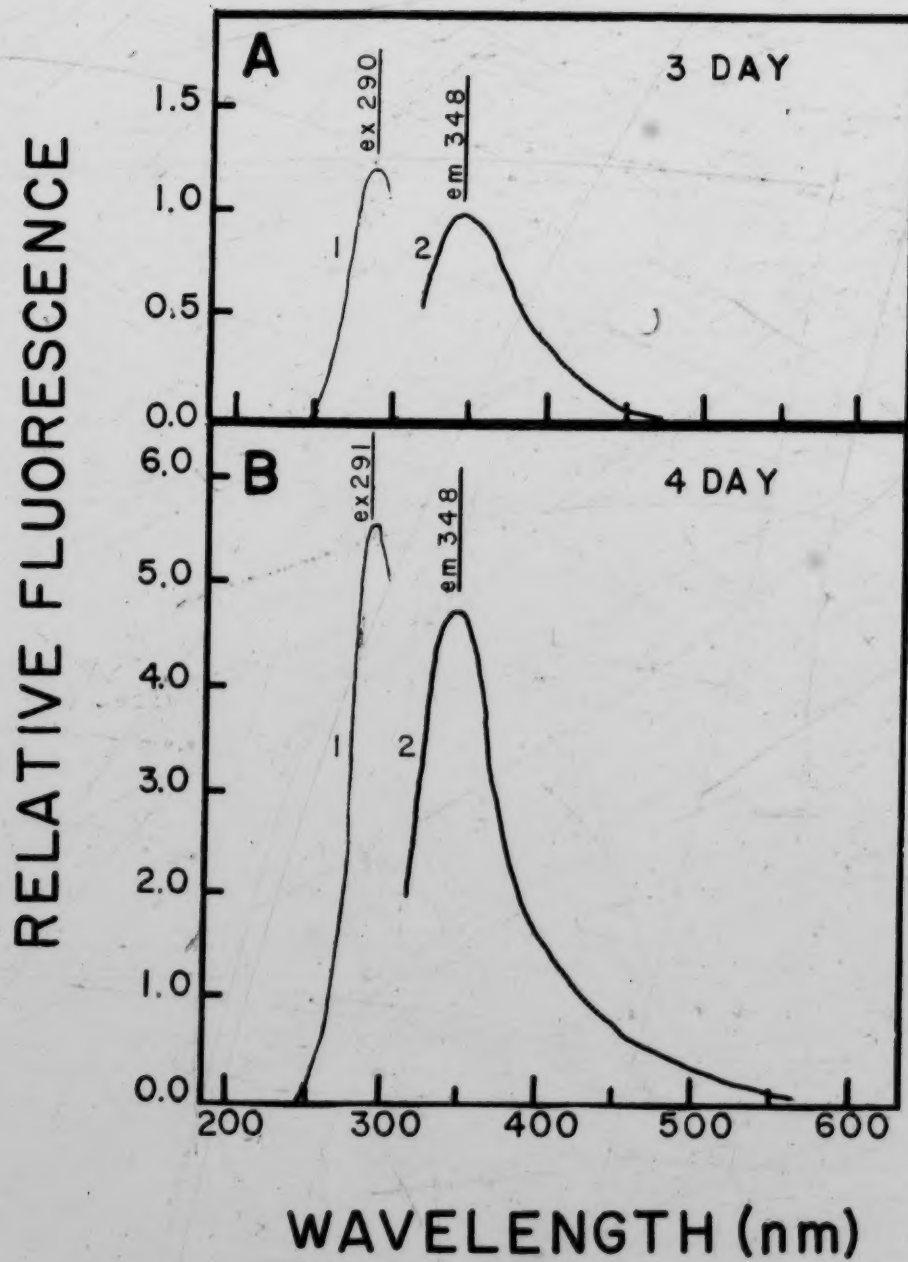
Fig. 12 (A-D). The progressive changes, during development, in the spectral patterns found in the ultraviolet region of fluorescence emission from the post-nuclear fraction of embryonic homogenates. The spectral patterns in this region of emission are presented from the embryonic homogenates prepared as described in the text. The light lines (—) on each figure represent the excitation spectrum (1); the heavy lines (—) represent the corresponding emission peak (2).

(A) shows the pattern obtained from an embryo of H-H. stage 19 (three-day);

(B) shows the pattern obtained from an embryo of H-H. stage 24 (four-day);

(C) shows the pattern obtained from an embryo of H-H. stage 27 (five-day); and

(D) shows the pattern obtained from an embryo of H-H. stage 29 (six-day).



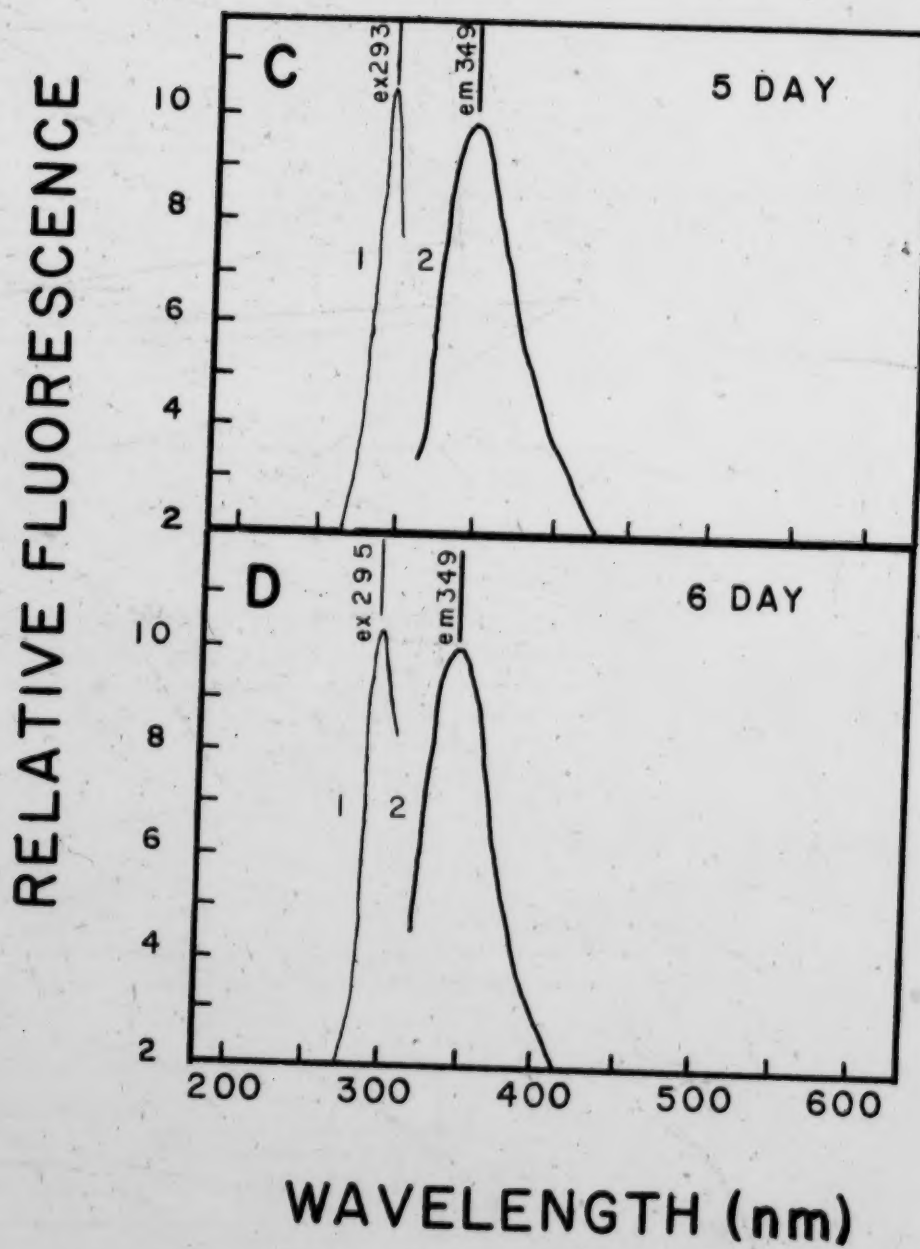


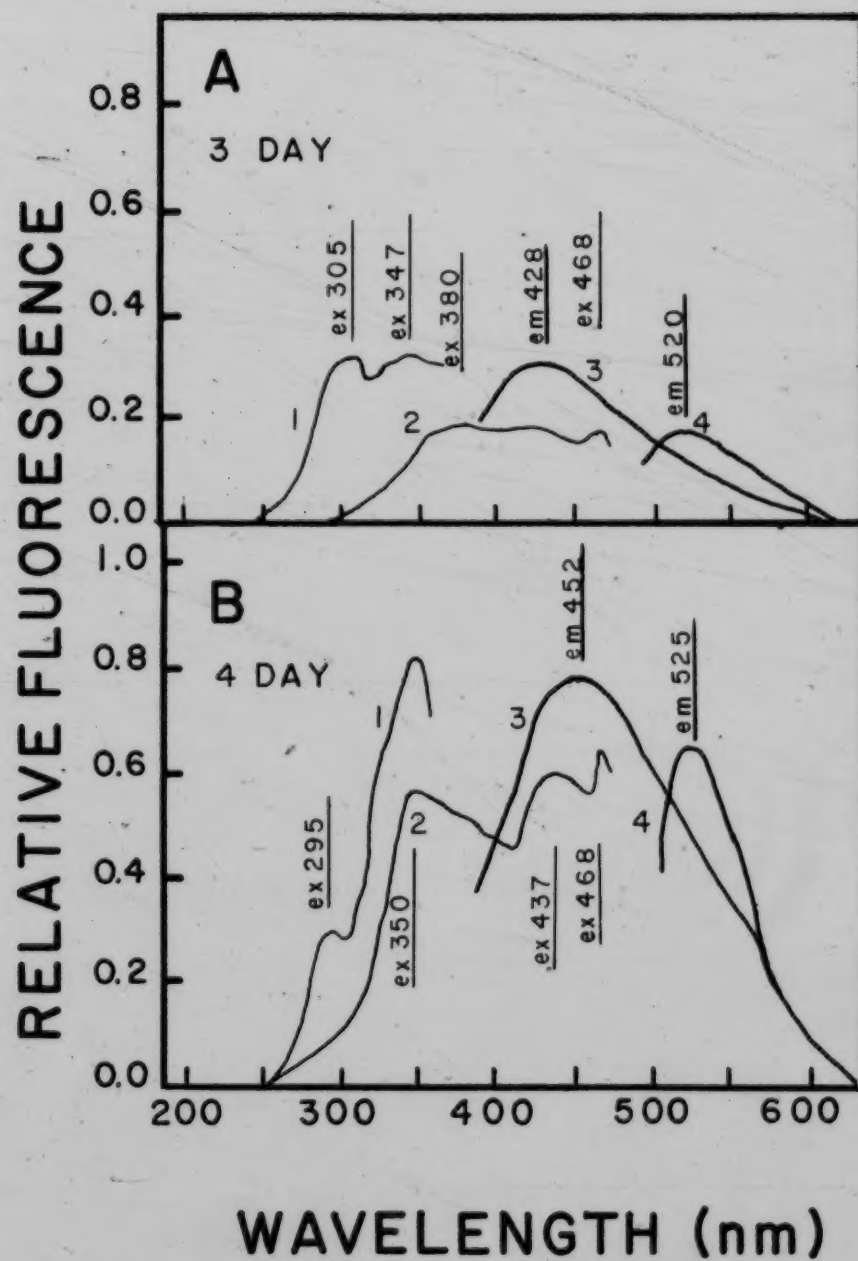
Fig. 13 (A-D). The progressive changes, during development, in the spectral patterns found in the visible region of fluorescence emission from the post-nuclear fraction of embryonic homogenates. The spectral patterns in this region of emission are presented from the embryonic homogenates prepared as described in the text. The light lines (—) on each figure represent the excitation spectra; the heavy lines (—) show the corresponding emission spectra.

(A) shows the spectral pattern obtained from the post-nuclear fraction of a stage 19 (three-day) embryo; spectrum (1) is the excitation spectrum of the 428nm emission peak (3); (2) is the excitation spectrum of the 520nm emission peak (4);

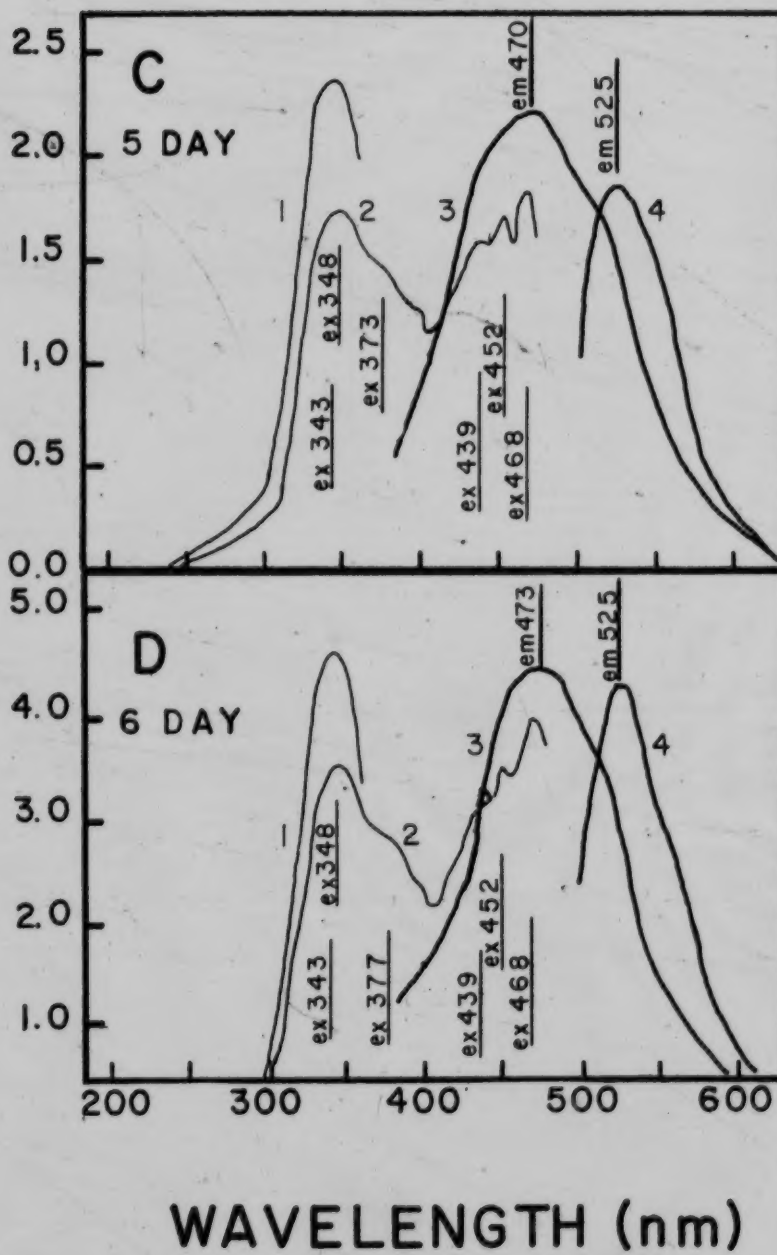
(B) shows the spectral pattern obtained from a stage 24 (four-day) embryo; (1) is the excitation spectrum of the 452nm emission peak (3); spectra (2) and (4) are as described for (A);

(C) shows the spectral pattern obtained from a stage 27 (five-day) embryo; spectrum (1) is the excitation spectrum of the 470nm emission peak (3); and spectra (2) and (4) remain the same;

(D) shows the spectral pattern obtained from a stage 30 (six-day) embryo; the spectra are as described in (C).



RELATIVE FLUORESCENCE



spectral shift described below.

The third emission peak present in the stage 16 embryo (430nm) undergoes a dramatic spectral shift as development proceeds. The excitation spectra shift from 350-360nm to 343nm, and the emission spectra shift from 428nm to 473nm from stage 19 to stage 30. This shift is accompanied by a 10- to 15-fold increase in fluorescence intensity.

Examining this shift closely, it is apparent that in the stage 19 embryo the 430nm emission peak has two excitation maxima, 305nm and 350-360nm. The excitation peak at near 300nm by itself causes fluorescence at about 350nm, which may then induce a secondary fluorescence near 450nm. Therefore it is reasonable to assume that the 450nm emission peak has a single excitation maximum at 350nm. In the stage 23 embryo (four-day), the excitation maximum shifts to 345nm with a shoulder at 295nm, the latter again due to the secondary fluorescence from the ultraviolet regions. The emission maximum is now at 452nm. In the stage 27 (five-day) embryo the excitation remains stationary at 343nm, but the emission is shifted to 470nm and the intensity is increased 7-fold. In the stage 30 (six-day) embryo the emission maximum is shifted slightly to 473nm, and, in comparison to the stage 27 embryo, the intensity is doubled. These results indicate that the spectral shift at 430-470nm region is completed by stage 27, and that the midpoint is apparently stage 23.

From the observations described above and summarized in Table IV, it is apparent that two of the emission peaks (348nm and 520nm) are relatively unchanged from stage 16 to 30. However, the third region of emission demonstrates a pronounced spectral shift (430nm to 470nm) over the same portion of development, with a midpoint in the shift of approximately stage 23. Such a shift could be due to the emergence of a new

TABLE IV. Changes in the excitation and emission maxima and relative fluorescence intensity seen in each emission region during early development. Individual embryos, grown under conditions "optimal" for normal development were each homogenized in 4ml buffer B, pH 7.8. The nuclei and cellular debris were spun out, and the excitation and emission spectra were recorded from the post-nuclear fraction of each. The relative fluorescence at each emission peak (given in parentheses) was calibrated and standardized by comparison to an acridine orange solution.

Day	(hour)	H.-H. stage	emission region #1 -(U V region)-		emission region #2 (visible region)		emission region #3	
			ex. (nm)	em. (nm)	ex. (nm)	em. (nm)	ex. (nm)	em. (nm)
3	(76)	19	290	348	305 and 350-360	428 (0.38)	380 and 468	520 (0.20)
4	(96)	23	291	348	shoulder-295 and max -350	452 (0.90)	350 and 468	525 (0.69)
5	(120)	27	293	349	343	470 (2.40)	348 and 468	525 (1.90)
6	(144)	30	295	349	343	473 (4.60)	348 and 468	525 (4.00)

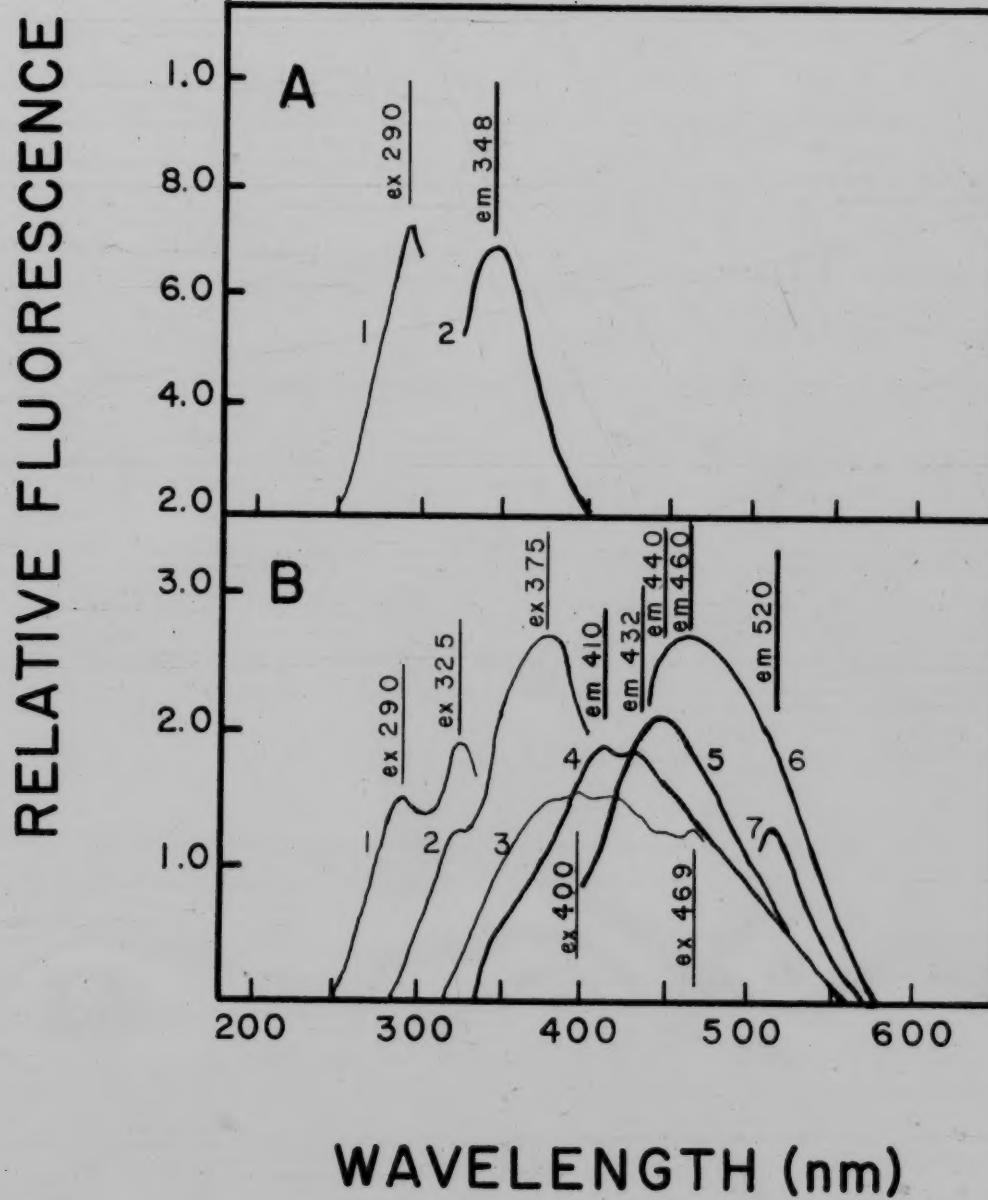
fluorescent substance or to a change in the condition of the fluorophore already present, such as coupling or uncoupling with macromolecules. These possibilities, among others, will be the subject of further investigation later in this thesis.

(ii) Fluorescence Spectra Characteristic to Egg Yolk: Prior to further analyses, it must be confirmed that the observed fluorescence is solely ascribable to the embryonic substance, not to contaminants from the yolk. In particular, the smaller specimen (stage 16 to 20) might have been contaminated by yolk substance, which is known to contain numerous fluorophores (for a review, see Romanoff, 1967). If so, the spectral shifts observed in the previous section may represent an artifact. To ensure that the observed spectral shifts represent events in embryos, samples of egg yolk from an unincubated egg, as well as from eggs incubated for three and six days, were examined, as described in Chapter 2-3 (ii).

The resulting spectra from each yolk sample were very similar. Therefore only one representative spectrum, that of the unincubated yolk sample, is presented (Fig. 14). There are four major regions of emission: (1) a peak excited at 290nm with an emission maximum at 348nm; (2) a peak excited at 325nm with a double emission maximum at 410nm and 432nm; (3) a peak excited at 375nm giving rise to a broad emission maximum from 430nm to 460nm; and finally (4) a small emission peak at 520nm with an excitation spectrum reminiscent of that peak in the stage 19 embryo.

Comparing the yolk spectra (Fig. 14) with the embryonic spectra (Fig. 12 and Fig. 13), the embryonic patterns and spectral shifts are clearly not due to simple yolk contamination. The yolk contains the dominant UV peak at 350nm found in the embryonic pattern, suggesting that a similar substance is present in both the yolk and the embryonic material.

Fig. 14 (A-B). The fluorescence spectral pattern demonstrated by whole egg yolk. Whole egg yolk from unincubated eggs was homogenized in buffer "B," pH 7.8, and the spectral patterns were recorded from the 12,000 x g supernatant. The light lines (—) represent the excitation spectra and the heavy lines (——) show the emission spectra. Fig. 14A presents the pattern in the U V range of fluorescence emission: spectrum (1) is the excitation spectrum for the 348nm emission peak (2). Fig. 14B presents the pattern in the visible range of fluorescence emission: (1) is the excitation spectrum for the 410-432nm emission peak (4); (2) is the excitation spectrum for the 440-460nm emission peak (5-6); (3) is the excitation spectrum which elicits the 520nm emission peak (7).



However, there are differences between the yolk and the embryonic patterns in the other two regions of emission found to dominate the embryonic spectra. The yolk contains several peaks in the 430-460nm region of emission, the range of emission in which the major spectral shift occurs in the embryonic pattern. However, the excitation and emission peaks found in this region of the yolk spectra do not coincide with those in the embryonic pattern, indicating that different substances are responsible. The third emission peak (525nm) found in the embryonic pattern is only a minor peak in the yolk spectrum. If the young embryo pattern is merely due to yolk contamination, this last peak should also be reduced in the embryonic pattern. Finally, the yolk spectral pattern is not altered, even after incubation for six days, while there are tremendous changes seen in the embryonic spectra over this time period, indicating that these fluorophores are embryospecific.

(iii) Effect of Incubation Temperature on the Time of Appearance of the Fluorescent Components During Normal Embryonic Development: The spectral changes, observed as development proceeds, may be the result of specific metabolic processes. Minor differences in incubation temperature could possibly lead to major changes in these metabolic processes, thereby affecting the spectral pattern obtained or the time in development at which the spectral shift occurs. Therefore, it was necessary to examine the effects of differences in incubation temperature on the spectral pattern. The spectra were recorded, as described in section (1), from individual embryos of H-H. stage 16 to 30, grown at four incubation temperatures, as described in Chapter 3-2 (ii). The results of this study are presented in Table V, listing the relative fluorescence at each emission maximum, standardized by comparison to an acridine orange solution, as described previously.

TABLE V. The comparison of relative fluorescence values obtained from embryos grown at four different incubation temperatures. Embryonic supernatants were prepared, examined, and the relative fluorescence values determined, as described in Table IV. The dotted lines indicate peaks which span several wavelengths (broad peak intermediates).

Temp. °C	hours	B.W.mg	C.L.mm.	Stage	Relative Fluorescence at peaks:				
					ex 290 em 350	ex 350-60 em 430-40	ex 350-60 em 450-60	ex 344- em 470	ex 470- em 525
35.5	76	11.0	1.40	16.0	0.665	0.447			0.165
	96	19.0	2.66	20.0	1.27	0.37			0.265
	120	47.0	4.00	22.0	2.56	0.45 ----	0.45		0.462
	144	163.0	7.05	25.5	6.2			1.7	1.55
36.5	76	10.0	1.84	17.0	0.524	0.333			0.214
	96	33.0	3.26	20.0	1.79	0.432			0.323
	120	65.0	4.50	23.5	4.96		0.775		0.76
	144	167.0	7.00	27.0	6.7			1.91	1.97
37.5	76	18.0	2.34	19.0	0.95	0.405			0.283
	96	52.0	4.27	23.0	3.48	0.516 ---	0.516		0.44
	120	189.0	7.45	27.5	8.7			2.39	1.88
	144	331.0	9.00	28.4	10.49			3.3	3.1
38.5	76	19.0	2.35	19.0	1.34	0.381			0.201
	96	69.0	4.60	23.5	5.76		0.891		0.685
	120	192.0	6.95	27.0	10.3			2.36	1.86
	144	440.0	9.00	29.0	10.32			4.6	4.0

Referring to Table V, the shift in emission peak from 430nm to 470nm occurs early, by 76 hours of incubation in the embryos grown at 38.5°C, 1°C above the temperature defined as optimal for normal development. This shift is only beginning on the fifth day (120 hours) of incubation in the group grown at 35.5°C, 2°C below the optimal temperature. When the relative fluorescence is plotted against incubation time (Fig. 15), embryos grown at 38.5°C demonstrate earlier increases in fluorescence intensity. The intensity reached in this group by 144 hours is greater than in those embryos grown at 2°C below the optimal temperature. Both these observations suggest that developmental changes in the embryonic spectral patterns are accelerated at the higher incubation temperatures but not altered. Discounting the incubation temperature and plotting the relative fluorescence at each maximum (Fig. 16) and its logarithm (Fig. 17) against the H-H. stage, there is a direct correlation between the fluorescence pattern and the stage. This correlation is unaffected by incubation temperature.

Referring to Fig. 17, the intensity of the 350nm emission peak increases logarithmically over the entire period of development investigated here, and the intensity of the 525nm emission peak increases logarithmically from stage 19. Since no large spectral shifts are observed in either of the above peaks, the fluorescence increases are probably due to an increase in the amount of these fluorophores per embryo. However, there is a very pronounced breakpoint in intensity at the time of the 430nm to 470nm transition, between stages 22 and 25. The intensity of the 470nm emission peak increases logarithmically after stage 25. The significance of such a breakpoint accompanying the spectral shift at this point in development will be examined later in this thesis.

Fig. 15 (A-C). Effect of incubation temperature on the rate of relative fluorescence increase at each of the three major emission peaks characteristic of early embryo whole homogenates. Embryonic homogenates were prepared, examined, and the relative fluorescence values determined, as described in the text (Chapter 2-3(vi)). Representative embryos were selected from the groups obtained in the temperature experiments described in Chapter 3: one for each day of incubation (three to six days) from each of four incubation temperatures: ($\blacktriangle$) 35.5°C; ($\bullet$) 36.5°C; ($\blacksquare$) 37.5°C; and ($\triangle$) 38.5°C.

(A) represents the relative fluorescence values at the 525nm emission peak (excited at 470nm);

(B) represents these values at the 430-470nm emission peak (excited at 350nm);

(C) represents these values at the 350nm emission peak (excited at 294nm), all plotted against incubation time in hours.

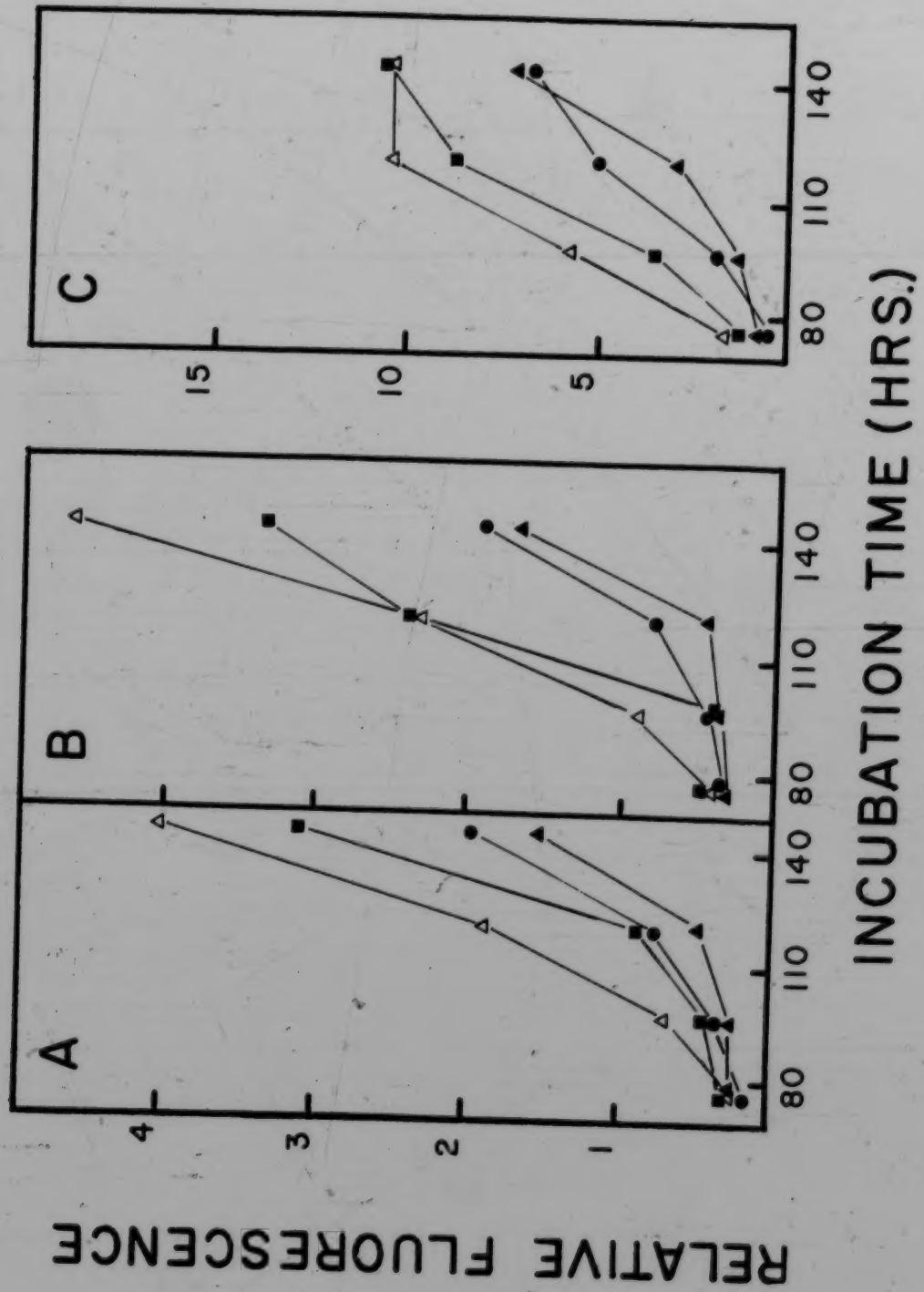


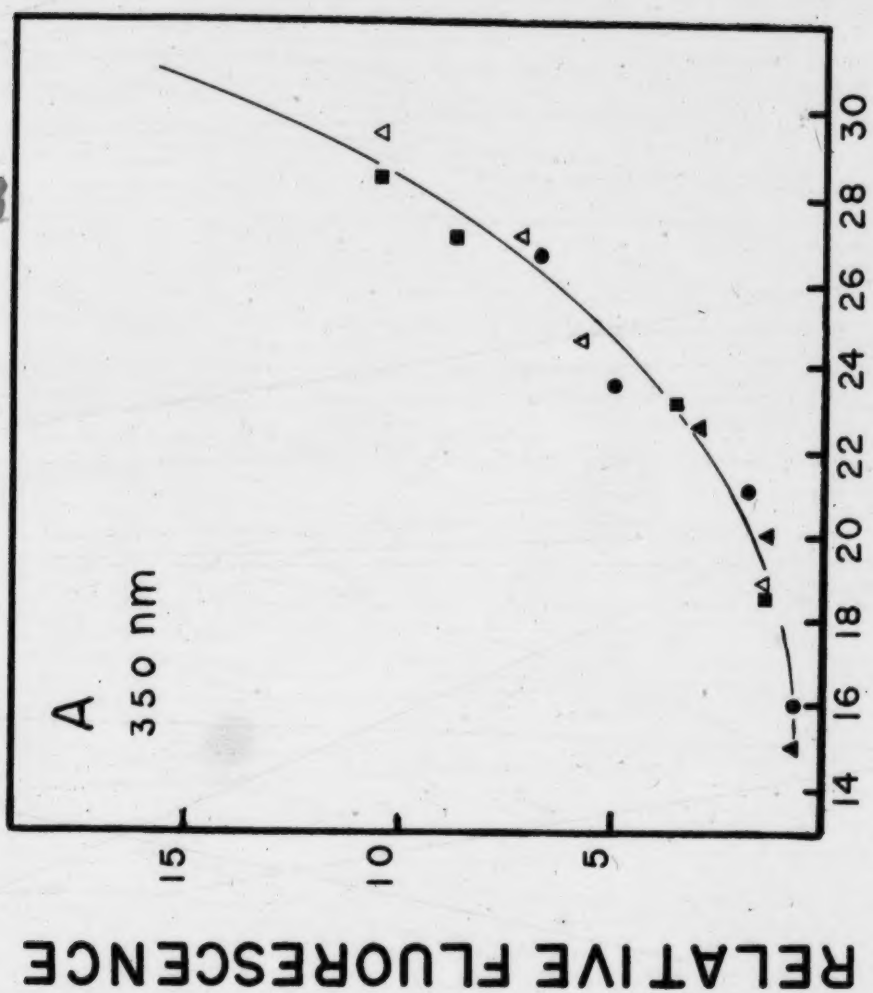
Fig. 16 (A-C). Effect of incubation temperature on the relationship between the stage of embryonic development and relative fluorescence at each emission peak characteristically found in embryonic homogenates. The values of relative fluorescence at each emission peak, given in Fig. 15, are plotted against stage of development of each individual embryo, as determined by the staging system outlined in Chapter 3. Each incubation temperature is identified as in the legend in Fig. 15.

(A) represents the relative fluorescence values at the 525nm emission peak (excited at 470nm);

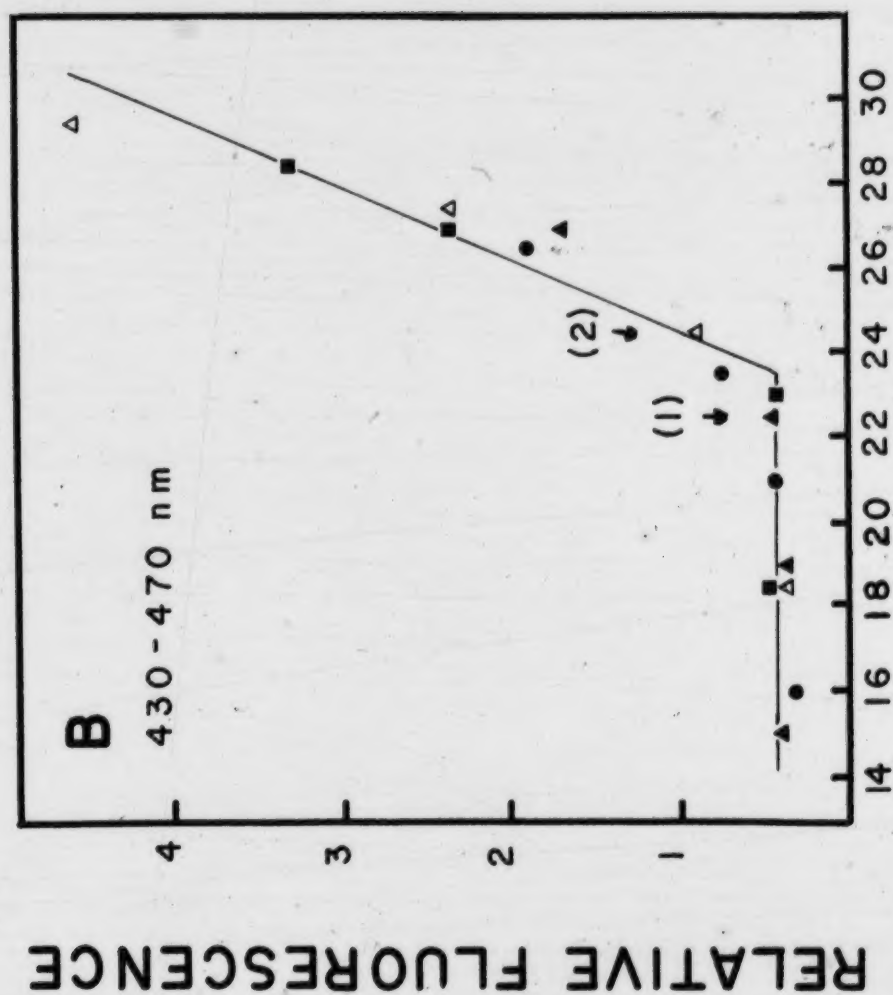
(B) represents those values at the 400-470nm emission peak (excited at 350nm);

(C) represents those values at the 350nm emission peak (excited at 294nm), all plotted against the H-H. stage of development.

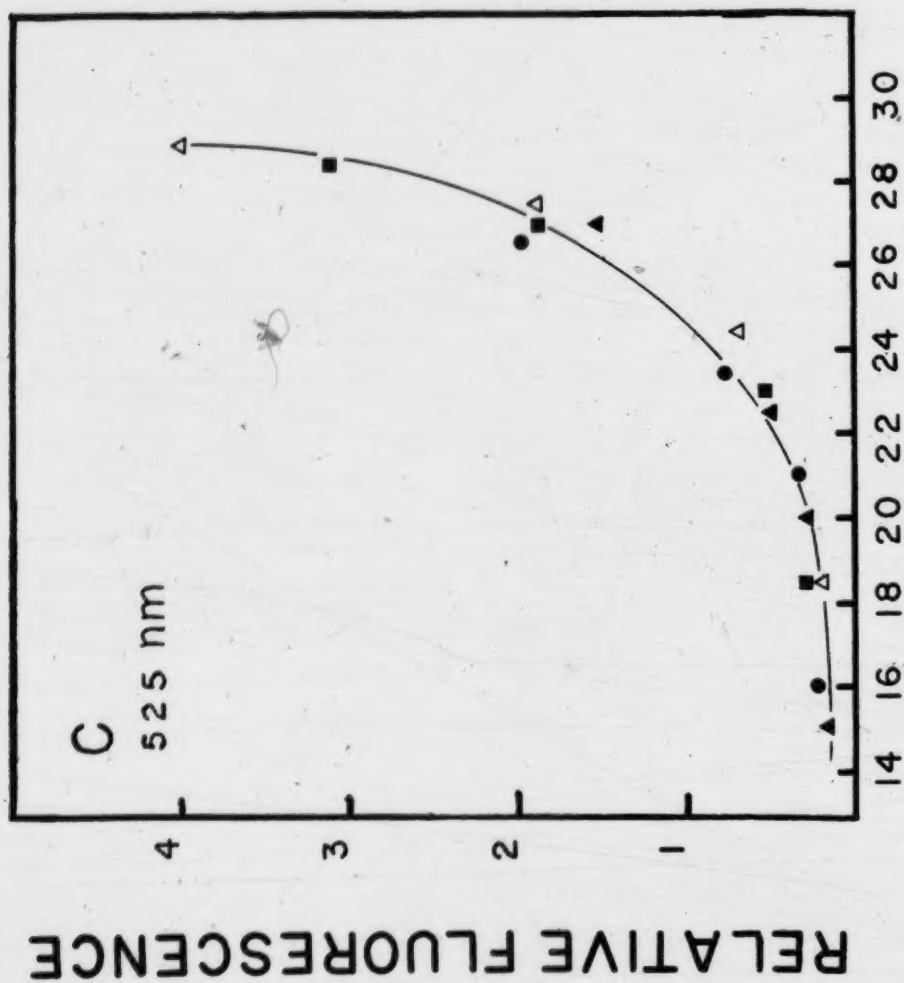
The arrows in (B) indicate approximately where the transition in emission peak occurs: (1) 430nm to 450nm; (2) 450nm to 470nm.



H. and H. STAGE

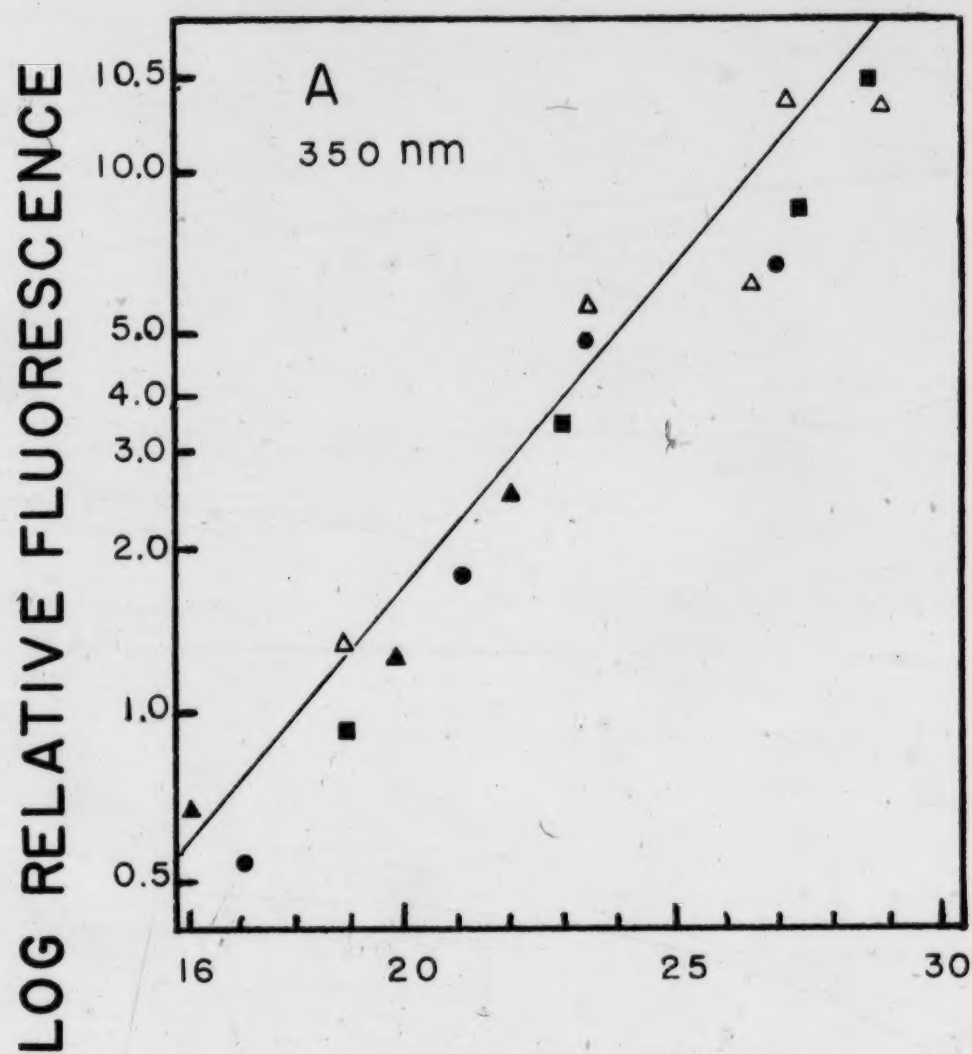


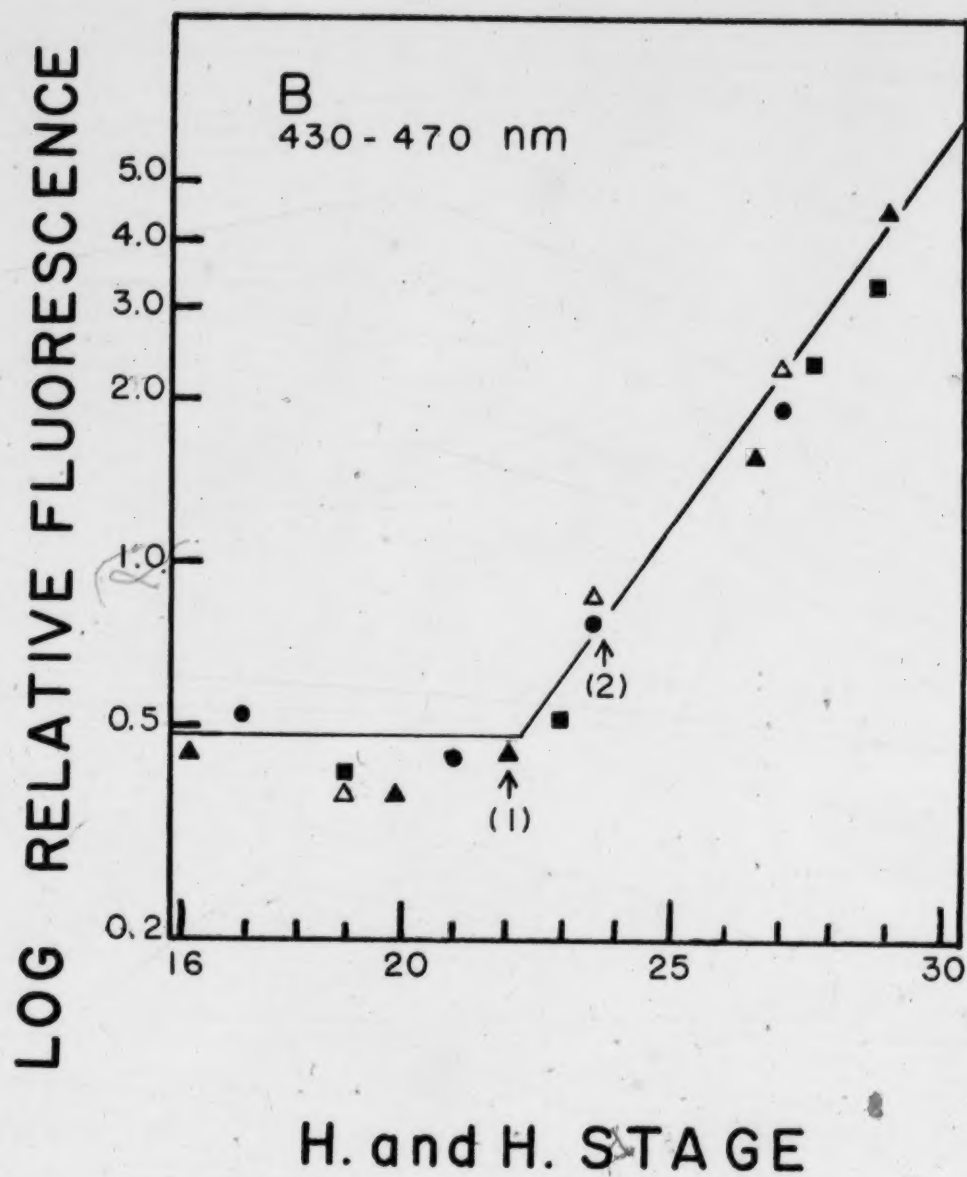
H. and H. STAGE

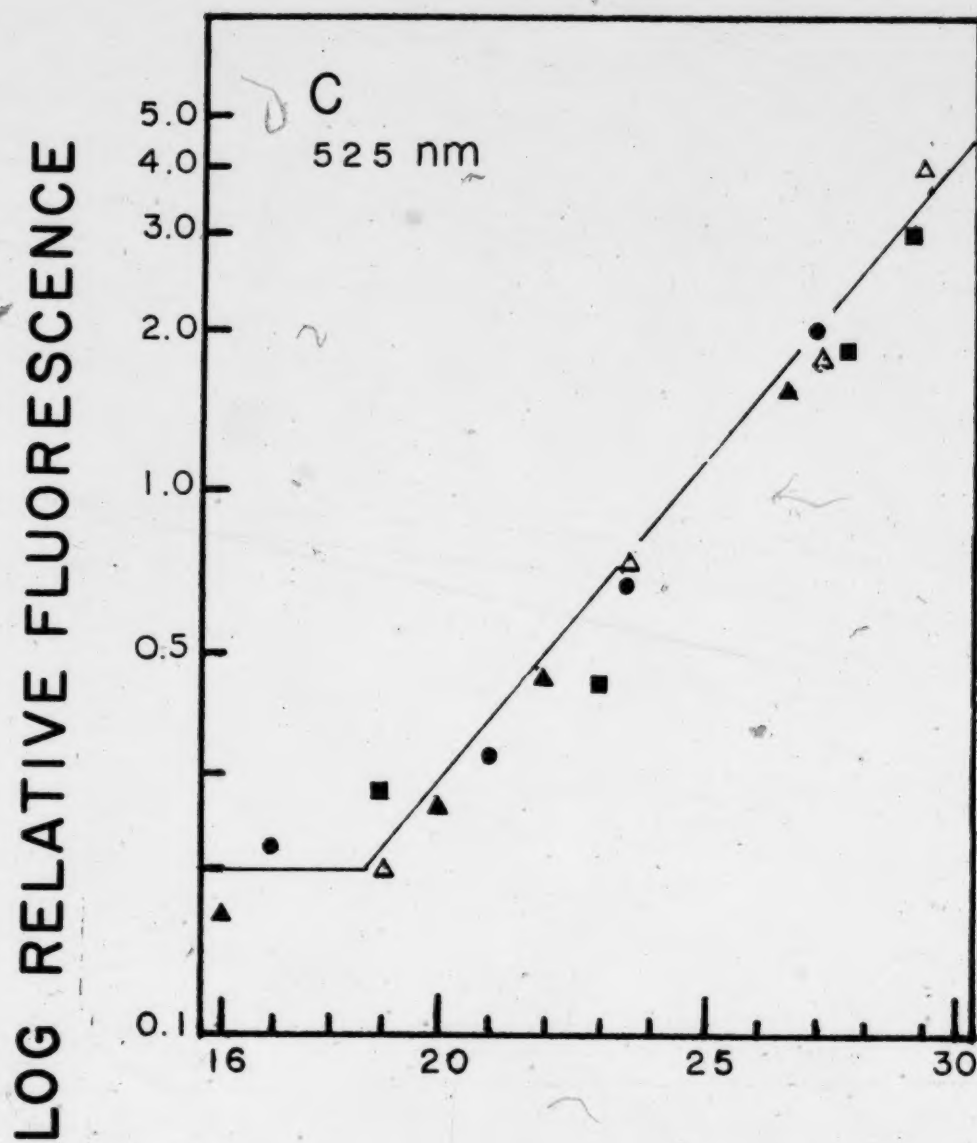


H. and H. STAGE

Fig. 17 (A-C). Semi-logarithmic repeat of Fig. 16. The relative fluorescence values of each emission peak were plotted semi-logarithmically against the stage of embryonic development. The legend and the description of (A-C) are the same as Fig. 16, except that the log values of relative fluorescence are plotted against stage of development.







H. and H. STAGE

In conclusion, the time of appearance and amount of each type of fluorescent substance is highly correlated with stage of development, and this relationship is unaffected by incubation conditions (i.e., temperature). Therefore, we are able to determine the precise time in development when specific changes occur at the molecular level.

4-3. Discussion and Summary:

As a means of analysis of complex tissues in aqueous extracts, fluorospectrophotometry is meaningful only if certain substances are present which dominate the fluorescence pattern (Deamer, 1974). Preliminary observation by Kato and Lauber (personal communication) and by Igarashi (personal communication) have revealed that there are dominant emission peaks in the spectra of chick embryos during the first week of development. Specific shifts in emission occur as development proceeds. In this chapter, in order to define the spectral characteristics seen at each stage and to determine when in development the spectral shifts occur, the fluorescence spectra were recorded from individual embryos of H-H. stage 16 to 30. By comparing the relative fluorescence of each emission peak to an acridine orange standard solution, it was possible to semi-quantitate the fluorescence intensity of each emission peak. Two of the three major emission peaks found to dominate the spectra merely increase in intensity as development proceeds. However, the third emission peak undergoes a spectral shift from 430nm to 470nm.

Because many fluorescent substances are present in the yolk (i.e., vitamin A₁, B₂, B₆) (for a review of egg yolk composition, see Romanoff, 1967), it was necessary to establish that the responsible fluorophores were truly embryonic components and not artifact. The fluorescence spectra of several yolk samples were examined and the patterns obtained

were compared to the embryonic spectra.

The yolk and the embryos were both homogenized in buffer "B", maintaining a constant environment. Therefore, if the same components were present in both, the spectral peaks should coincide. In the UV region, similar peaks were seen in the yolk and the embryonic patterns. However, the excitation and emission peaks found in the yolk spectrum did not coincide with the embryonic peaks in the region of emission which shifts as development proceeds. This suggests either that different compounds are present in the yolk and the embryo, or that the same fluorophores are present in both, but that the molecular environment is different in the yolk and the embryo, thereby altering the fluorescence characteristics. The third emission peak found in the embryonic pattern is a minor component in the yolk, and simple yolk contamination would not result in such a pronounced 525nm emission peak in the embryonic pattern. Finally, the yolk spectrum remains unchanged after six days of incubation, indicating that the spectral shifts seen in the embryonic pattern are due to the change in embryo-specific fluorophores.

Having established that the embryonic fluorescence is not a yolk contamination, we proceeded to pinpoint the stage in development when specific changes occur in the spectral pattern. Because many cofactors, metabolic products, and by-products have the ability to fluoresce (Seltzer, 1967; Kahan, 1967; Clark, 1975; Pennock, 1962), it is possible that the changes in the spectral pattern of the embryo are due to specific metabolic changes. Many of these metabolic changes (e.g., enzymatic reactions) found in the early stages of development may be quite sensitive to temperature differences. Slight variation from the optimal incubation temperature may induce changes in these processes. It was therefore necessary to determine the effect, if any, of

differences in incubation temperature on the spectra obtained at various stages of development.

The spectral patterns associated with each stage of development, as assessed by the staging system discussed in Chapter 3, remain the same regardless of incubation temperature. Thus, the correlation between the stage of development and the spectral pattern is unaffected by minor differences in incubation conditions. This correlation allows precise determination of the time in development when specific changes occur in the fluorescence spectra.

The increase in fluorescence intensity, at the 350nm and 525nm emission peaks, follows closely the body weight curve seen in Chapter 3 (Fig. 5), suggesting that these substances merely increase in amount as development proceeds. However, the other region of emission is more complex. Accompanied by a noticeable breakpoint in intensity after stage 22, there is a shift from a 430nm emission peak at stage 22 to a 452nm peak at stage 23 to 24, and a final shift to a 470nm emission peak by stage 25. These results suggest that a specific change occurs at this point in development.

This shift could be the result of a differential uptake of yolk substance(s), beginning at this stage. The yolk contains a variety of fluorescence components and, as seen in this chapter, displays a complex fluorescence pattern in which individual spectral peaks are difficult to distinguish. However, if there is a selective incorporation into the embryo of certain fluorophores from the yolk, the true excitation and emission maxima of these fluorophores may then be identified.

The initiation of a certain cellular process or the beginning of synthesis of a specific cellular component may lead to a shift in the spectral pattern. The products and/or by-products formed by an embryo-

specific synthesis, for example, may demonstrate different fluorescence characteristics than does the parent compound(s).

Finally, certain fluorescent molecules, either taken up from the yolk or produced in the embryo itself, may be incorporated into cellular matrices or cellular organelles as development proceeds. Upon incorporation, the configuration of these molecules may be altered, leading to spectral shifts. The configurational changes could create fluorescence from originally non-fluorescent components.

In conclusion, by examining the fluorescence pattern obtained from the post-nuclear supernatants of individual embryos from H-H. stage 16 to 30, we have been able to detect a specific developmental change at the molecular level in the form of a spectral shift occurring between stage 22 and 25. Several explanations have been presented for the fluorescence behavior observed. Such a shift could be the result of a differential uptake of specific yolk components; or the result of the formation of fluorescent products or by-products by an embryo-specific synthesis; or possibly the result of incorporation of certain fluorophores into cellular organelles or matrices, thereby altering the electronic environment of the fluorophores and causing a change in the spectral pattern exhibited. In order to determine the cause of this spectral shift and to examine its biological significance, attempts will be made in Chapter 5 to isolate and identify the molecules involved.

CHAPTER 5

SUB-CELLULAR LOCALIZATION AND CHARACTERIZATION OF THE FLUOROPHORES IN EMBRYONIC HOMOGENATES

5-1. Introduction:

In the preceding two chapters, we established the fluorescence patterns obtained from homogenates of individual embryos of H-H. stages 16 to 30. This enabled us to detect specific changes in the pattern and pinpoint exactly when in development these changes occur. The most pronounced change was a red-shift in one of the emission peaks (430nm to 470 nm) between H-H. stage 22 and 25. Two approaches may now be taken in order to examine the biological significance of such a spectral shift during this portion of development.

First, by utilizing histological techniques combined with fluorescence microscopy, it may be possible to determine the location within the embryo of the fluorophores responsible for this spectral change. This approach would provide a clue as to what morphological or anatomical changes these fluorophores are involved with. In our study, however, such an approach was not feasible due to technical limitations. As seen in Chapter 4, the intensity of fluorescence exhibited by the homogenate of individual embryos is so low that the spectra must be recorded at sensitivity levels where the Raman spectrum of water is an interfering factor. At such a low level of intensity the fluorescence from a sectioned embryo would not be detectable by a fluorescence microscope.

Because of the limitations imposed by a histological approach, a second approach was adopted in our study, and the final phase of this thesis involves the sub-cellular localization and subsequent characterization of the fluorophores in embryonic homogenate. Determination of sub-cellular location could provide information as to the type of

sub-cellular components these fluorophores are associated with, and possibly information as to the cause of the spectral shift. Fractionation of the embryonic homogenates may separate non-fluorescent embryonic materials from the fluorescent materials, thus providing the initial step in purification of the fluorophores. Once the fluorophores responsible for the spectral shift are isolated and identified, it may be possible to examine the role these substances play in morphological and/or physiological changes known to occur during this period of development.

In order to determine the sub-cellular location of the embryonic fluorophores, a fractionation procedure was devised, based on the method utilized by Igarashi (personal communication). A homogenate of three-day (H-H. stage 16 to 20) embryos was prepared to examine the location of the 430nm emission substance, and a homogenate of six-day (H-H. stage 28-30) embryos was prepared to examine the location of the 470nm emission substance. Upon fractionation of these homogenates, the majority of fluorescence at both 430-440nm and 470nm was recovered in the soluble-protein fraction, indicating that the fluorophores are either freely soluble or bound to a soluble molecule.

In hope of isolating the 470nm emission substance, the soluble-protein fraction from a six-day embryo was subjected to a series of chromatographies. These attempts were unsuccessful, however, due to the instability of the 470nm emission peak. Under the conditions employed in the chromatography, the 470nm emission peak shifts to a stable peak at 440nm, producing a pattern which mimics the characteristics of the three-day embryo. This suggests that a single fluorophore is involved. Because of the stability of the 440nm emission peak, we chose to focus our study on this component in hope of finding clues to the identity of the components involved and, possibly, clues to the cause of the spectral shift.

In pursuing the identity of the 440nm emission substance, methods were employed to separate the embryonic substances in terms of differential solubility. Three forms of the 440nm emission substance were found: (1) an acid-soluble, chloroform-insoluble form; (2) an acid-insoluble, chloroform-soluble form; and (3) an acid-insoluble, chloroform-insoluble form. These results suggest that this fluorophore is bound to several types of carrier molecules. In order to clarify the type(s) of carriers involved as well as to purify the fluorophore, portions of each fraction described above were subjected to enzymatic digestion. These digestions were followed by a re-examination of the solubility. The results suggest that there are protein, lipoprotein, and possibly glycolipoprotein molecules associated with the 440nm emission fluorophore. This fluorophore apparently derives its stability from association with these molecules. Upon release from this complex, the fluorophore becomes unstable; the 440nm emission peak disappears and several new emission peaks are seen. Therefore we were not able to purify this component for identification of the chromophore. This must be the object of further study. However, the fact that the fluorophore(s) involved in the spectral shift is apparently bound to several types of carrier molecules is of interest, and the significance of this observation will be the main topic of the discussion in this chapter.

During this study, the identification of the two other fluorophores (responsible for the emission peaks at 350nm and 525nm) was carried out concurrently. The fluorophore excited at 290-295nm, with an emission peak at 350nm, is attributable to tryptophane residues in the soluble proteins. The region of fluorescence excited by a double peak of 382nm and 470nm, and displaying an emission peak at 525nm, is attributable to riboflavin or its derivatives.

5-2. Results:

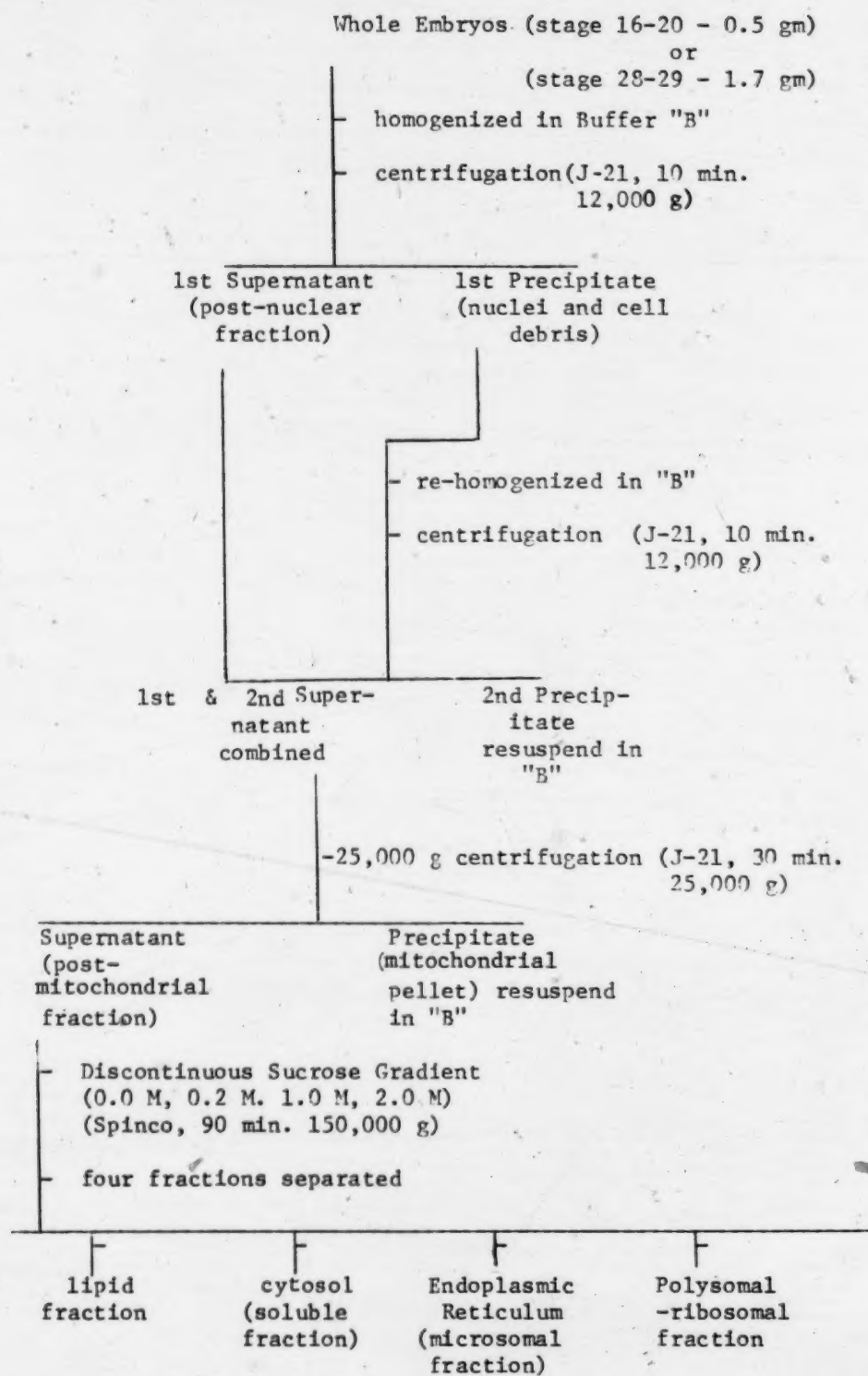
(i) Fractionation of Sub-Cellular Components in Homogenates of Whole

Embryos: Before the sub-cellular location of the embryonic fluorophores could be determined, it was necessary to fractionate the homogenates. The centrifugation procedure designed for this purpose is outlined in Fig. 18. Each sample of embryonic tissue to be fractionated was first homogenized in 16 ml of buffer "B" and then subjected to the following procedure:

(a) Differential centrifugation: The homogenate was spun at $12,000 \times g$ in the Beckman J-21 centrifuge for 10 minutes at 4°C , removing the unbroken cell fragments and the nuclei. The resulting pellet was re-homogenized in 3 ml of "B" and spun again. The second pellet was re-suspended in 3 ml of buffer "B" and placed on ice. The post-nuclear supernatants were combined and brought to a final volume of 12 ml with buffer "B". This solution was then spun at $25,000 \times g$ in the J-21 for 20 minutes at 4°C to remove the mitochondria and any remaining cellular debris. The pellet was suspended in 2 ml of buffer "B" and placed on ice. The post-mitochondrial supernatant was then subjected to further separation as described in (b) below and Fig. 18.

(b) Discontinuous sucrose gradient ultracentrifugation: Buffered sucrose solution (pH 7.8) was added to the post-mitochondrial supernatant, bringing it to a final concentration of 0.2 M sucrose and volume of 13 ml. This supernatant was divided into two equal portions (6.5 ml) and placed in 12 ml cellulose centrifugation tubes. Each sample was underlaid with 1.5 ml of 2.0 M sucrose in buffer "B" and 1.5 ml of 1.0 M buffered sucrose, and then was overlaid with 2.5 ml of buffer "B", pH 7.8. The gradients were spun at 4°C for 90 minutes at $150,000 \times g$ in a Spinco L3-50 preparative ultracentrifuge. Four fractions were collected by puncturing the bottom of the cellulose tube and were placed on ice in the dark until measurement

Fig. 18. Fractionation method utilized in separation of the sub-cellular components in whole embryo homogenates. The details of this procedure are given in section (iv). Aliquots of each fraction obtained by the above procedure were taken, and the total volume was recorded accurately for later determination of the total relative fluorescence in each portion. All of the above procedures were performed in dim light, and the samples were kept in the dark at 4°C until analyses.



of the fluorescence. The fractions and their contents are as follows:

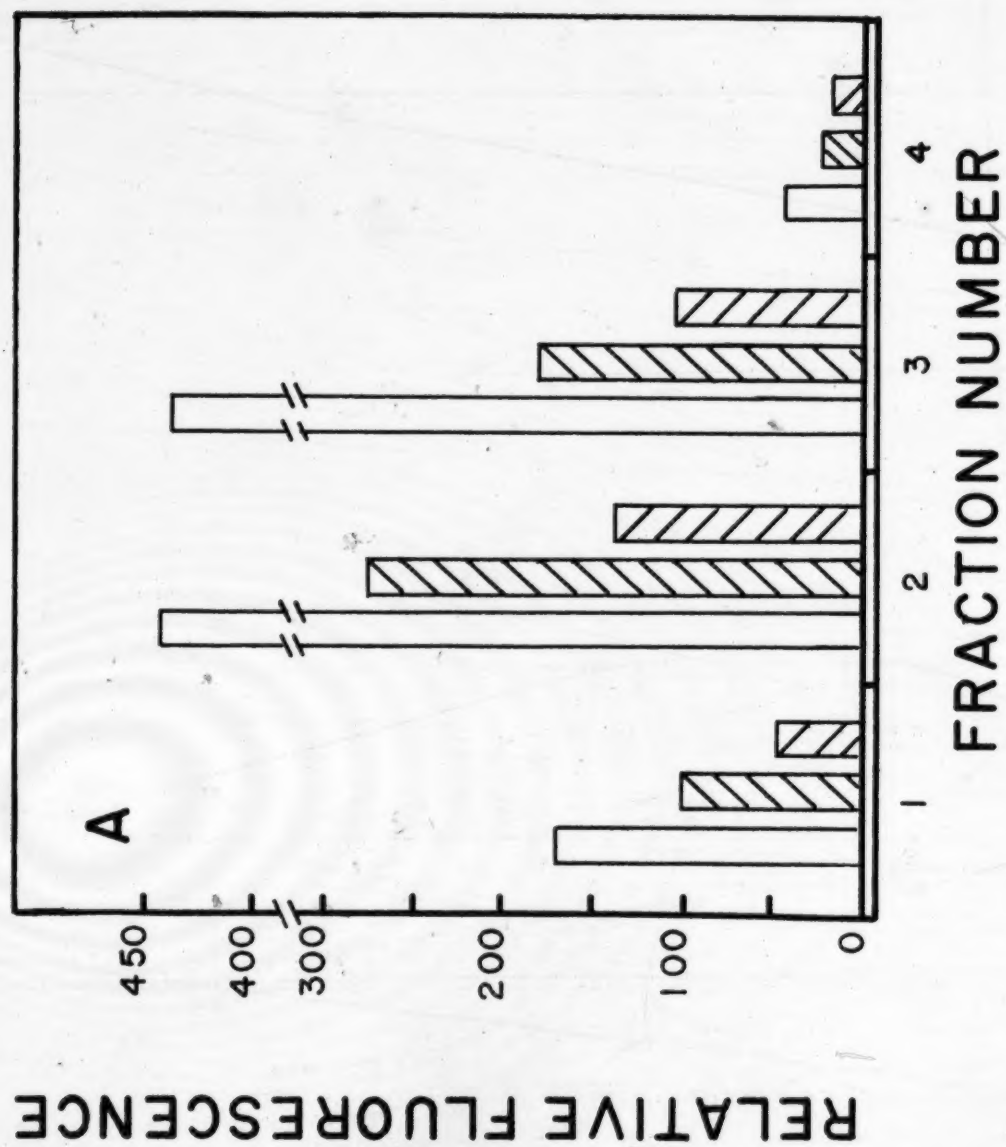
(1) the top buffer layer + the 0.0 M - 0.2 M interface = the lipid layer; (2) the 0.2 M layer = the soluble-protein layer containing the cytoplasmic fluid and the soluble serum components; (3) the 0.2 M - 1.0 M interface = microsomal fraction; and (4) the 1.0 M interface material = the polysomal fraction.

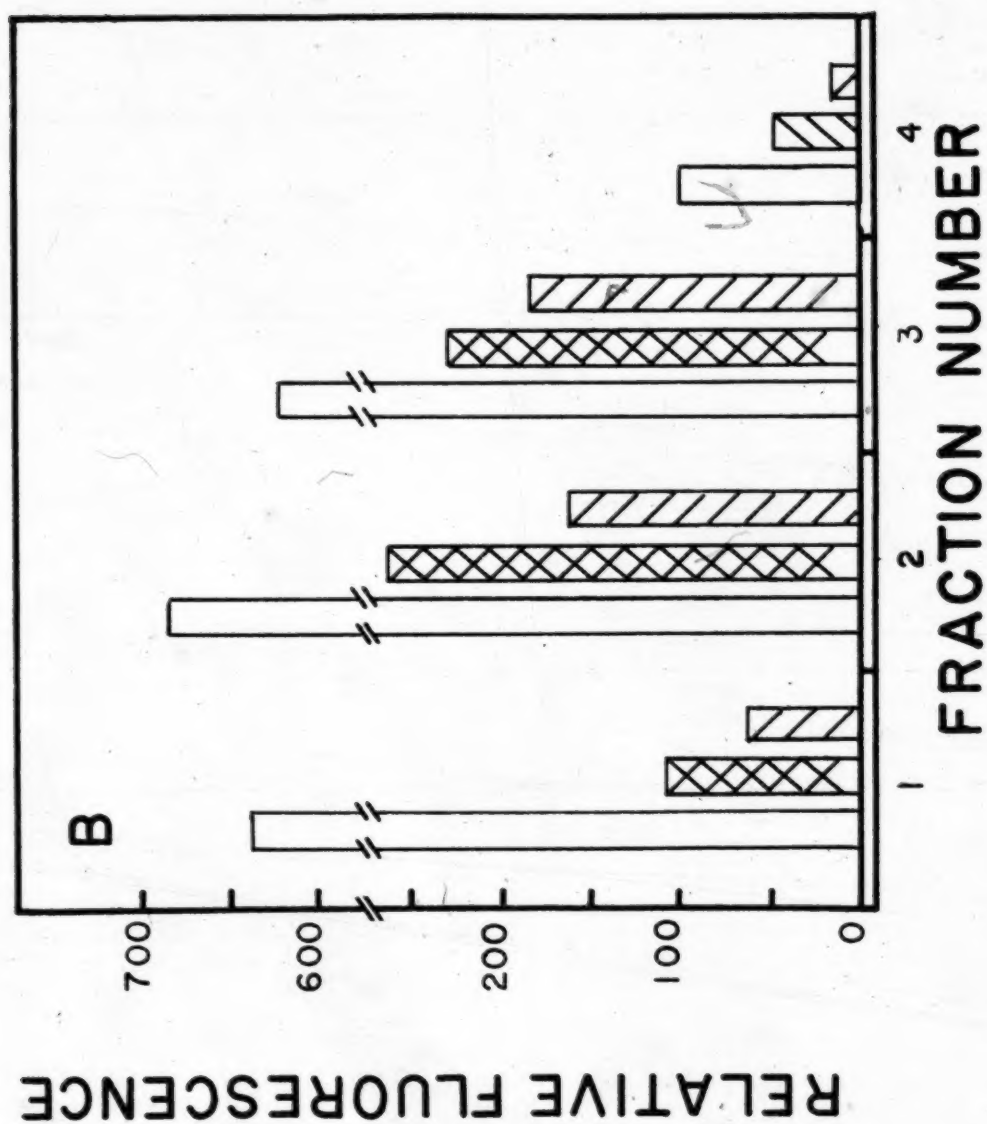
(ii) Sub-cellular Location of Fluorescent Substances: Two samples of embryonic material were used: a 0.5 gm sample of pooled three-day (H-H. stage 16-20), containing the 430nm emission substance, and a 1.7 gm sample of pooled six-day (H-H. stage 28 to 30) embryos, containing the 470nm emission substance. These were homogenized and fractionated by the procedure outlined in section (i). The fluorescence spectra were recorded from each of the resulting fractions. The major excitation and emission peaks seen are: an excitation peak at 295nm yielding a major emission peak at 350nm; an excitation of 350-360nm yielding a 430-440nm emission in the three-day embryo sample; an excitation of 350-360nm yielding a 465-470nm emission in the six-day embryo sample; and an emission peak at 525nm with an excitation pattern dependent upon the age of the embryos fractionated. These peaks are identical to those in the spectra of individual embryos, indicating that the fractionation methods do not affect the molecular environment of the fluorophores with respect to their ap-structure.

The intensity of fluorescence at each emission maximum in each fraction obtained by differential centrifugation (section (i)-a) is presented in Fig. 19 (A and B). When the fluorescence exhibited by the post-mitochondrial supernatant is compared with the fluorescence exhibited by the nuclear and mitochondrial pellets, it is evident that most of the fluorescent material is in the post-mitochondrial supernatant. Igarashi

Fig. 19 (A). The total relative fluorescence in each fraction obtained by separating homogenates of three-day embryos by differential centrifugation. Twenty-six embryos (500mg total wet weight) of H-H. stages 16 to 20 (approximately three-days) were pooled and homogenized in buffer "B." This homogenate was fractionated as described in 5-2(1). Fraction 1 contains the nuclei and unbroken cells; Fraction 2 is the post-nuclear fraction; Fraction 3 is the post-mitochondrial supernatant; Fraction 4 represents the mitochondrial fraction. The total relative fluorescence at each emission peak was determined by multiplying the relative fluorescence obtained from a 1 ml aliquot by the total volume of each fraction. The fluorescence emission peaks are: emission 350nm (excited at 295nm)  ; emission 440 (excited at 350nm)  ; and emission 535nm (excited at 470nm) .

Fig. 19 (B). The total relative fluorescence in each fraction obtained by separating homogenates of whole six-day embryos by differential centrifugation. Four embryos (1,700 mg total body wet weight) of H-H. stages 28 to 30 (approximately six-days) were pooled and homogenized in buffer "B." This homogenate was fractionated as described in 5-2(1). The total relative fluorescence was determined as above, and the legend remains the same as in Fig. 19 (A), except that an extra emission peak of 470nm (excited at 350nm) was added .





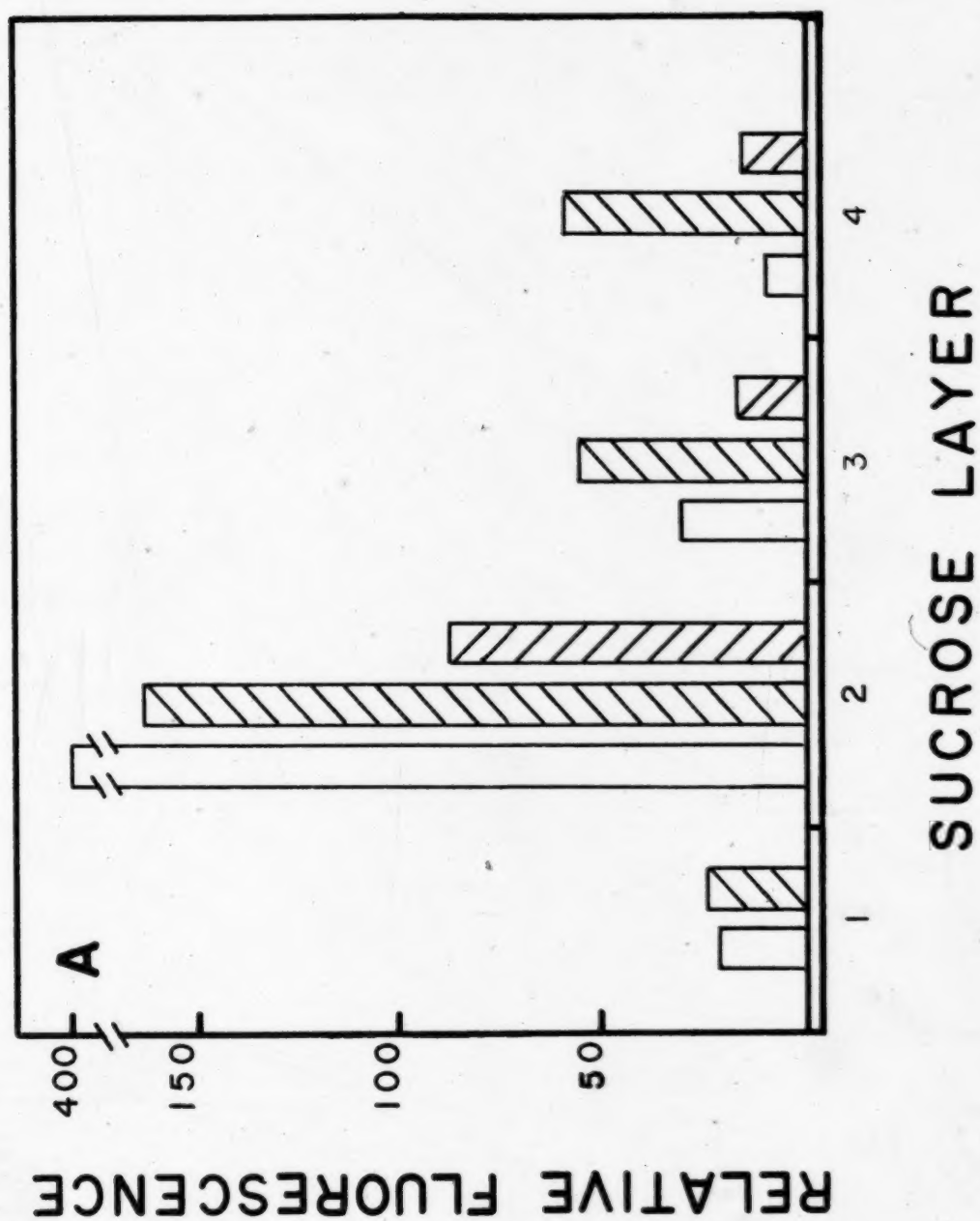
(personal communication), utilizing a buffer solution containing 0.25 M sucrose and PMSF, designed to prevent nuclear and mitochondrial leakage, had also observed that the nuclear and mitochondrial pellets contain only minor amounts of fluorescence. Therefore it may be assumed that the minor amounts of fluorescent material in these fractions are probably due to contamination from the post-mitochondrial supernatant. The quantity of fluorescence at 350nm in the nuclear pellet of the six-day embryo is possibly due to the fact that the older embryos contain large amounts of structural protein, not readily solubilized by our extraction procedure and hence appearing with the nuclear pellet.

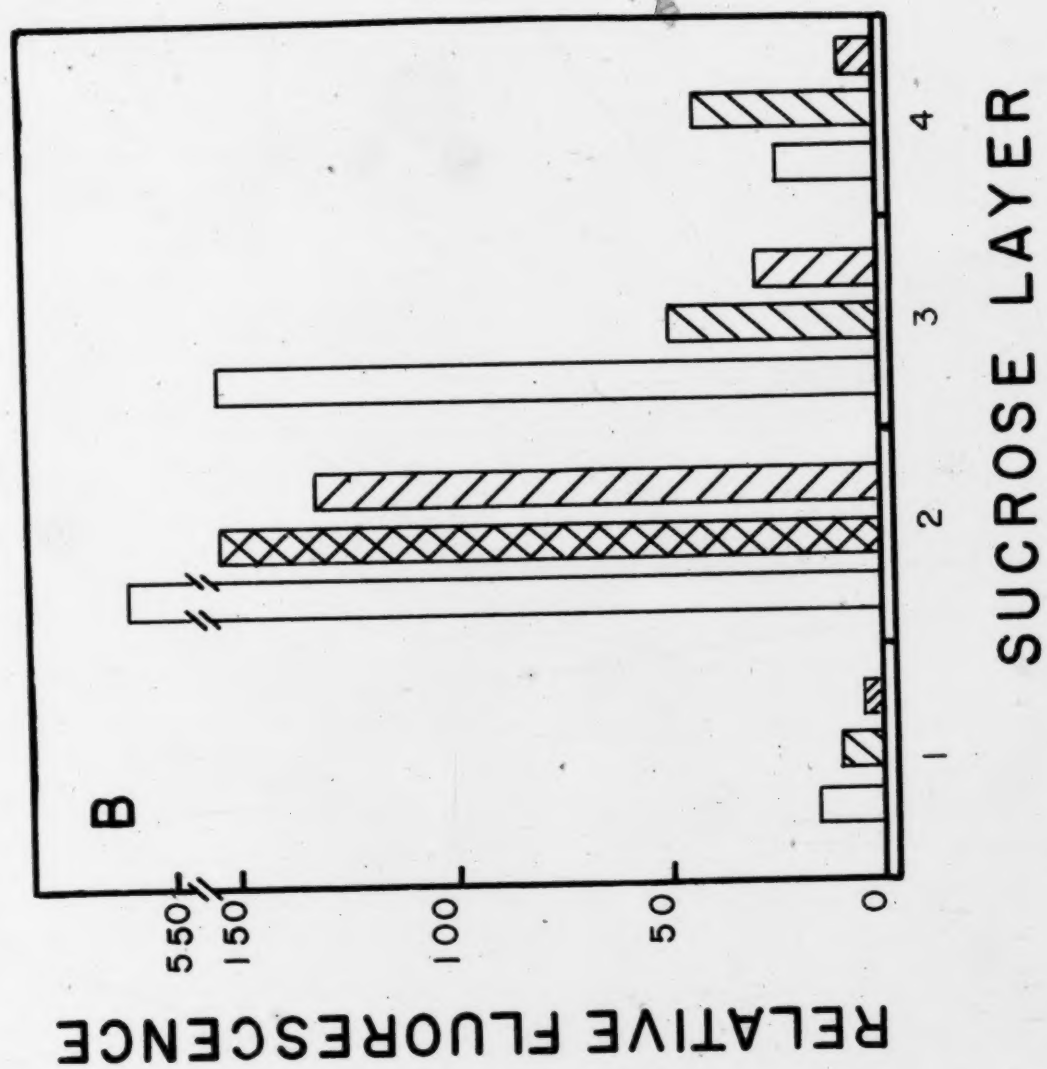
The intensity of fluorescence at each emission maximum in each fraction of the discontinuous sucrose gradient (section (i)-b) is presented in Fig. 20 (A and B). The majority of fluorescence at each emission peak is found in the soluble-protein fraction in both the three-day and six-day samples, with the exception of a rather large amount of fluorescence at 350nm in the microsomal layer of the six-day embryo. The lipid and microsomal layers of both three-day and six-day embryos contain minor amounts of fluorescence at 440nm and 525nm. This fluorescence may, however, be due to contamination of the soluble-protein layer, and this question will be examined in more detail later in this chapter. The polysomal layer also exhibits some fluorescence. There is some question as to the origin of this fluorescence, and this must be re-examined in another study.

The main finding in this fractionation experiment is that the fluorophores responsible for the major emission peaks, and for the shift in the spectral pattern, are localized in the soluble-protein (cytosol) fraction. These fluorophores may be freely soluble in aqueous fluid themselves, or they may be solubilized by binding to a "carrier" molecule

Fig. 20A. The total relative fluorescence in each fraction obtained by separating three-day embryo post-mitochondrial supernatant on a discontinuous sucrose gradient. The post-mitochondrial fraction of the three-day homogenate obtained by differential centrifugation was fractionated further, as described in 5-2 (1). The four layers obtained are: (1) a lipid layer; (2) a soluble-protein layer; (3) an endoplasmic reticulum heavy layer; and (4) a polysomal layer. The total relative fluorescence is determined as in Fig. 19, and the legend is unchanged from Fig. 19A.

Fig. 20B. The total relative fluorescence in each fraction obtained by separating the post-mitochondrial supernatant from six-day embryos on a discontinuous sucrose gradient. The post-mitochondrial fraction from six-day embryos obtained by differential centrifugation was fractionated further as described in 5-2 (1). The four layers obtained are as above, and the legend follows that of Fig. 19B.



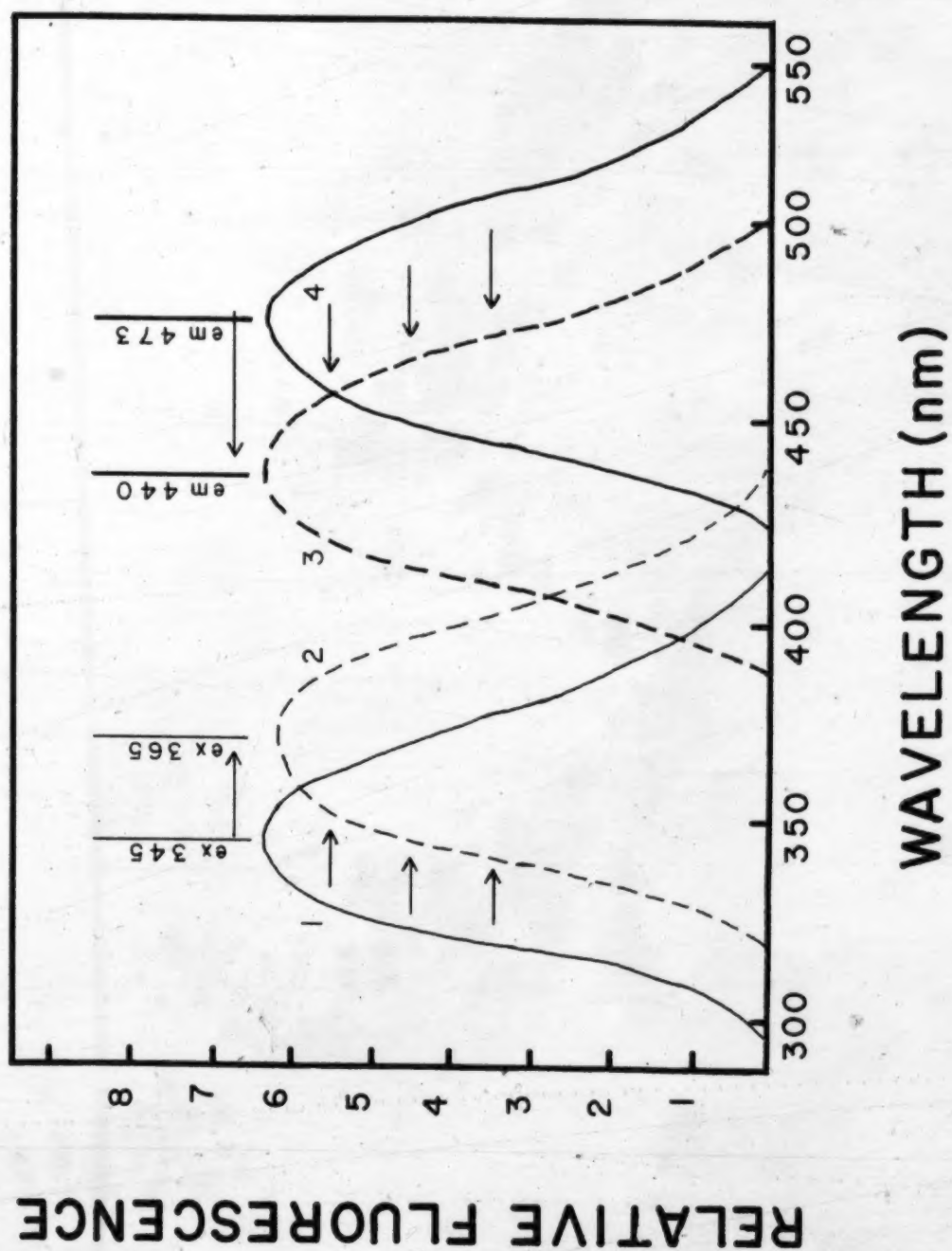


which is soluble. However, it should be cautioned here that the methods employed do not allow the separation of "free serum substances" from "soluble cellular components". Differentiation between these two locations must await further examination.

(iii) Instability of the 470nm Emission Component: Having established the sub-cellular location of the embryonic fluorophores, we attempted to isolate and characterize those fluorophores involved in the spectral shift, from 430nm to 470nm seen between H-H. stages 22 and 25 (Chapter 4). We first attempted to purify the substances responsible for the 470nm emission peak, utilizing chromatography. Portions of the soluble-protein fraction of a homogenate of six-day embryos, prepared as described in section (ii), were subjected to separation by chromatographic procedures outlined in Chapter 2-3 (xiii). These attempts were unsuccessful, however, because no fluorescence at 470nm was found in the effluent. The component was either bound so tightly to the columns that it could not be removed or its spectral characteristics were altered by these procedures.

In an attempt to determine the cause of the loss of fluorescence at 470nm, we examined the stability of this emission peak. Material containing the 470nm emission component was exposed to a variety of conditions (described in Chapter 2-3 (x)) which, among other effects, cause the denaturation of soluble proteins. The 470nm emission peak was found to shift to a stable peak at 440nm (Fig. 21), confirming an earlier observation by Igarashi (personal communication). These results suggest that possibly the 470nm emission peak is due to a fluorophore bound to a carrier molecule. By removal of the carrier by chromatography or upon denaturation of the carrier, the fluorophore would be exposed to the surrounding environment, resulting in a change in the spectral characteristics. Such behavior is not uncommon to fluorescent substances, many of

Fig . 21. The spectral shift in the 470nm emission peak of a six-day embryo homogenate upon storage at -20°C for one year. The solid line (——) represents the original excitation peak (1) which elicits an emission at 470nm (4). The broken thin line (-----) represents the final (stable) excitation spectrum (2) and its emission at 440nm (3). Upon prolonged storage at low temperature of either the intact six-day embryo or the 12,000 x g supernatant (post-nuclear fraction), the above shifts are seen in the excitation and emission maxima. The scale used was expressed as arbitrary fluorescence units, as we were observing only qualitative changes.



which exhibit totally different spectral patterns in bound and free form (e.g., vitamin A in bound and unbound form, Peterson, 1971). Also the fact that the 470nm emission peak shifts to a 440nm emission peak, mimicking the peak characteristic of a three-day embryo, suggests that the spectral shift seen as development proceeds is due to a change in binding of a single type of fluorophore.

(iv) Isolation and Characterization of the Solubility of the 440nm

Emission Compound: In the previous section, our efforts to purify the 470nm emission component were not successful due to the unstable nature of the fluorescence peak, which was found to shift to 440nm under a variety of conditions. However, the 440nm emission produced is quite stable, permitting continued attempts at characterization and identification of the fluorophore which is responsible. In order to obtain sufficient material for this investigation, a post-mitochondrial supernatant of six-day embryos, which demonstrated a large peak at 440nm due to the shift from 470nm, was fractionated as described in section (i). The soluble-protein fraction obtained was then subjected to a series of treatments, outlined in Fig. 22 and Fig. 23, designed to separate the components based on solubility.

The acid-soluble and acid-insoluble substances were separated by a TCA precipitation, described in Chapter 2-3 (xvii). The proportions of relative fluorescence at 440nm in the acid-soluble form, compared to that in the acid-insoluble form, varied from between 1:1 to 1:2, depending on the length of time allowed for the precipitate to settle. Three possible explanations for the differential solubility demonstrated by this component are: (1) the fluorophore is acid soluble, a portion of which is bound to an acid-insoluble component (such as acidic protein); (2) the fluorophore is an acid-insoluble substance, a

Fig. 22. Flow chart outlining the experimental procedure utilized in characterization of the 440nm emission component from the soluble portion (0.2 M fraction of a D.S.G.) of the six-day embryonic homogenate. The details of each step are presented in the text. The fractions containing the majority of the fluorescence at 440nm are inclosed in boxes.

Soluble Fraction (0.2 M Fraction
S.D.G.)

- TCA (5% final volume)
- spun 32,000 x g 10 minutes (J-21)

Precipitate

1/2 to 3/4 of total fluorescence at
440nm recovered here

sample split into four portions

Supernatant

1/4 to 1/2 of total
fluorescence at 440nm
recovered here
(continued in Fig. 23)

- 1.-----extracted with water-saturated
chloroform

-vortex, spun 2,000 x g 10 minutes (J-21)
three layers

Buffer layer

Denatured layer
all of fluorescence
at 440nm recovered here

Chloroform layer

- 2.-----digested with α -chymotrypsin at 37°C for 16 hours

-TCA extraction (440nm fluorescence recovered in the precipitate)

-precipitate extracted with water-saturated chloroform

-vortex, spun 2,000 x g 10 minutes (J-21)
three layers

Buffer layer
1/4 of fluorescence at
440nm recovered here

Denatured layer

Chloroform layer
3/4 of fluorescence at
440nm recovered here

- 3.-----digested with lipase at 37°C for 16 hours

-TCA extraction (most of 440nm fluorescence recovered
in the precipitate)

-precipitate extracted with water-saturated chloroform

-vortex, spun 2,000 x g 10 minutes (J-21)
three layers

Buffer layer

Denatured layer
3/4 of fluorescence at
440nm recovered here

Chloroform layer

4.-----digested with hyaluronidase 37°C for 16 hours

-TCA extraction (most of 440nm fluorescence recovered in the precipitate)

-precipitate extracted with water-saturated chloroform

-vortex, spun 2,000 x g 10 minutes (J-21)

three layers

Buffer layer

Denatured layer

Chloroform layer

3/4 of fluorescence at
440nm recovered here

Fig. 23. Flow chart outlining the experimental procedures and behavior of the 440nm emission component found in the TCA-soluble fraction obtained in the procedure outlined in Fig. 22. The details of each step are presented in the text.

The first TCA soluble fraction in the procedure outlined in Fig. 22 was split into four fractions and subjected to

1. ----- extraction with water-saturated chloroform--majority of fluorescence at 440nm remains in the aqueous supernatant.
2. ----- pH was adjusted to pH 12.0 spun 25,000 x g (J-21) 10 min.-- majority of fluorescence at 440nm remains in the alkaline soluble form.
3. ----- digestion with hyaluronidase at 37°C for 16 hours-- chloroform extracted--majority of fluorescence at 440nm remains in the aqueous supernatant.

portion of which is bound to an acid-soluble component (such as a basic protein); or (3) the fluorophore is bound to both acid-soluble and acid-insoluble components, thus is not in a free form at all. To explore these possibilities several experimental approaches were undertaken.

First, the solubility characteristic of the 440nm emission substance present in the acid-soluble form (in the TCA supernatant) were examined. A 3 ml portion of the TCA supernatant, adjusted to pH 7.8, was extracted with water-saturated chloroform according to the procedure described in Chapter 2-3 (xv). The majority of the 440nm emission fluorescence remained in the aqueous phase (Fig. 23 #1), indicating the component is not a lipid-soluble form. The aqueous layer was then adjusted to pH 12.0 by titration with 0.1 N NaOH, and this solution was spun at 4°C for 10 minutes at 25,000 x g in a Beckman J-21 centrifuge to remove the basic proteins (Fig. 23 #2). The precipitate, resolubilized in buffer "B" (pH 7.8), was found to contain only a minor amount of fluorescence at 440nm, with the majority still in a soluble form, indicating that the fluorophore is not associated with a basic protein. Finally, in order to determine if the 440nm emission component is solubilized by association with a polysaccharide molecule, a 3 ml portion of the supernatant, adjusted to pH 7.8, was digested with 100 μ g of hyaluronidase (suspended in 10 μ l of distilled water). This enzyme attacks the glyoxyl (C -1 --- O) bonds between sugar residues and a polysaccharide molecule, breaking the parent molecule into tetra- and oligosaccharides (Meyer, *et. al.*, 1960). The digest was incubated at 37°C for 16 hours and then extracted with water-saturated chloroform as described earlier. The majority of the 440nm emission substance was again found in the aqueous layer (Fig. 23 #3). The results of the procedure described above suggest that the 440nm emission component may

be in a free form in the TCA supernatant. This possibility must be subjected to further scrutiny.

The solubility behavior of the acid-insoluble form of the 440nm emission component was then examined. A 1 mg portion of the TCA precipitable material obtained earlier was re-suspended in 3 ml of Tris-HCl (pH 7.8) as described in Chapter 2-3 (xvii), and this solution was subjected to a chloroform extraction (Fig. 22 #1). The majority of the fluorescence at 440nm emission was recovered in the interface material, which contains denatured protein. This suggests that the fluorophore is bound to an acid-insoluble protein. To test this hypothesis, another portion of the TCA insoluble material was suspended in buffer and was subjected to proteolytic digestion. Pronase, a non-specific protease, was originally used for this digestion (Matsubaba and Feder, 1971). However, the pronase contained impurities which exhibited fluorescence at 440nm. Therefore, α -chymotrypsin, an enzyme which hydrolyzes peptide bonds at tyrosine and tryptophane residues, was used for the digestion because it contained very little fluorescence (Hess, 1971). One hundred micrograms of α -chymotrypsin (in 10 μ l of distilled water) was added to a portion of TCA insoluble material, suspended in 3 ml of Tris (pH 7.8), and this digest was incubated for 16 hours at 37°C (Fig. 22 #2). After digestion the fluorescence at 440nm was again found to be insoluble in TCA. Upon extraction with chloroform, the fluorescence was recovered in the chloroform layer. Thus, proteolytic digestion apparently releases the 440nm emission component from a carrier protein. The chloroform solubility acquired by this component upon release from this carrier suggests that the free form of the 440nm component is soluble in organic solvents. However, this conflicts with the observed behavior of the 440nm emission component in

the TCA soluble fraction. A second possibility is that the fluorophore is bound to a carrier lipoprotein, which becomes soluble in chloroform upon digestion of the protein moiety. In order to clarify the type of binding involved, several more enzymatic digestions were undertaken.

Another portion of the original TCA insoluble material, in 3 ml of Tris buffer, pH 7.8, was digested by the addition of 100 μ g of lipase (in 20 μ l of distilled water) (Fig. 22 #3). This lipase enzyme hydrolyzes neutral fats to fatty acid and glycerol (Desnuelle, 1972). The digest, after incubation at 27°C for 16 hours, was precipitated with TCA, and the fluorescence at 440nm remained in an acid-insoluble form. This material was subjected to a chloroform extraction, and again the major amount of fluorescence was found in the interface material. Thus, lipase digestion has no effect on the solubility of this fluorophore, as might be expected if the fluorophore were bound to a lipoprotein. The protein moiety of the molecule, if an acidic protein, would remain acid-insoluble and chloroform-insoluble after lipase digestion.

Finally, in order to determine if polysaccharide components were involved, a portion of the TCA insoluble material, in 3 ml of buffer, was subjected to digestion with hyaluronidase (100 μ g in 10 μ l of distilled water) (Fig. 22 #4). Following digestion the majority of the fluorescence at 440nm remained in an acid-insoluble form. However, upon extraction with chloroform the fluorescence was now found in the aqueous layer instead of in the interface material. Thus, the hyaluronidase digest apparently caused a release of the fluorophore, implying that the actual carrier may be a glycolipoprotein.

The results obtained to this point in our investigation indicate that there are two forms of the 440nm emission component in the soluble-protein fraction of the embryonic homogenate: (1) an acid-soluble form

which may be a free form, although it could be bound to a small peptide or a low-density lipoprotein not easily precipitated or extracted; and (2) an acid-insoluble form which is more prevalent and apparently associated with a lipoprotein and/or possibly even a glycolipoprotein "carrier" molecule.

As noted in section (ii), the microsomal and lipid fractions of the embryonic homogenate contain some fluorescence at 440nm. The presence of the 440nm emission material in these fractions may be due to simple contamination from the soluble protein fraction. It could also be due to the association of the 440nm emission component with some sub-cellular components contained in these fractions. Therefore, it was of interest to determine the solubility characteristics of the 440nm emission component in these fractions.

The microsomal and lipid fractions obtained by fractionation of the six-day embryo homogenate were subjected to the same separation procedures which had been utilized in the examination of the soluble-protein components. The steps involved, and the results obtained, are presented in Fig. 24. Because the 440nm emission component displays identical behavior in both the microsomal and the lipid fractions, only the results from the examination of the microsomal layer are presented.

A TCA precipitation again yielded acid-soluble and acid-insoluble forms of the 440nm emission component, with the majority of fluorescence in the insoluble form (approximately 3/4). The proportion again varied slightly, depending on the time allowed for precipitation to occur. The acid insoluble material was subjected to chloroform extraction (Fig. 24 #1); and equal amounts of fluorescence at 440nm were recovered in the interface material (containing denatured proteins.), and the chloroform layer (containing lipid-soluble material). However, if the acid-insoluble

Fig. 24. Flow chart outlining the experimental procedure utilized in characterization of the 440nm emission component in the microsomal fraction (1.0 M fraction D.S.G.) of the six-day embryonic homogenate. The details of each step are presented in the text. The fractions containing the majority of the fluorescence at 440nm are enclosed in boxes.

Microsomal Fraction (1.0 M Fraction D.S.G.)

-TCA (5% final volume)

-32,000 x g 10 minutes (J-21)

Precipitate

-2/3 of total fluorescence at 440nm
recovered here

-split into three fractions

Supernatant

-1/3 of total fluorescence at 440nm recovered
here

1.-----extraction with water-saturated chloroform

-vortex, spun 2,000 x g 10 minutes (J-21)
three layers

Buffer layer

Denatured layer1/2 of fluorescence
at 440nm recovered
hereChloroform layer1/2 of fluorescence
at 440nm recovered
here2.-----digested with α -chymotrypsin 37°C for 16 hours-TCA extraction (all of the fluorescence at 440nm recovered here
in the precipitate)

-precipitate extracted with water-saturated chloroform

-vortex, spun 2,000 x g 10 minutes (J-21)
three layers

Buffer layer

Denatured layer

Chloroform layer

3.-----digested with hyaluronidase and lipase 16 hours

-TCA extraction (most of the fluorescence at 440nm recovered
in the precipitate)

-precipitate extracted with water-saturated chloroform

-vortex, spun 2,000 x g 10 minutes (J-21)
three layers

Buffer layer

Denatured layer

Chloroform layer

material was first subjected to digestion with α -chymotrypsin, followed by TCA precipitation and chloroform extraction (Fig. 24 #2), all of the fluorescence at 440nm was recovered in the chloroform layer. These results suggest that, in addition to the lipoprotein-bound form seen in the soluble-protein layer, there is a lipid or membrane-bound form in the microsomal layer.

In order to examine the possibility of a membrane-bound form, a portion of the acid-insoluble material, suspended in 3 ml of Tris-HCl (pH 7.8), was subjected to a combined digestion with hyaluronidase (100 μ g in 10 μ l of water) and lipase (100 μ g in 20 μ l of water). This solution was incubated for 16 hours at 37°C (Fig. 24 #3). This treatment would be expected to break up structural components such as those found in the reticular layer, thus exposing a lipid-bound fluorophore to chloroform extraction. After digestion, this solution was precipitated with TCA; and the acid-insoluble material, which contained the 440nm emission substance, was extracted with chloroform. The fluorescence at 440nm was recovered in the chloroform layer. This indicates that the digestion either liberates the fluorophore to a free form soluble in chloroform, or that the digestion is not extensive enough to completely destroy the lipid or membrane carrier, thereby granting chloroform solubility to the fluorophore.

The following procedure was employed to examine the question further as well as to attempt to purify the 440nm emission fluorophore. A new homogenate of six-day embryos was prepared and fractionated as described earlier. The microsomal layer was extracted with chloroform, the chloroform layer evaporated, and resuspended in buffer "B", pH 7.8. This solution was subjected to extensive lipase digestion. Three 100 μ g portions of lipase (each suspended in 20 μ l of water) were added at

8-hour intervals during incubation at 37°C. After digestion the 440nm emission peak became unstable, preventing further attempts to determine the solubility characteristics or to purify the free form of this fluorophore.

The results of this last experiment indicate that the fluorophore is associated with a lipid or membrane component in the microsomal fraction. It is of interest that the excitation and emission characteristics of this fluorophore are not shifted more than 5 to 10nm when bound to different carrier molecules or when released from the lipid carrier. Such fluorescence behavior suggests this fluorophore is at least partially exposed to the surrounding environment. This behavior backs up the theory that we are dealing with carrier molecule complexes.

In conclusion, the solubility behavior, the response to enzymatic digestions, and the fluorescence behavior of the 440nm emission substance suggest that there are at least three forms of this substance in the embryonic homogenate: (1) an acid-soluble form possibly bound to a short polypeptide or a low density lipoprotein not easily precipitated or extracted by chloroform; (2) an acid-insoluble form in the soluble-protein fraction bound to a lipoprotein and/or a glycolipoprotein; and (3) an acid-insoluble form bound to lipid components in the reticular layer. The instability of the free form of the fluorophore indicates that it may be necessary to purify the fluorophore while bound to its carrier molecule. Once this complex is purified, the components may then be separated and identified individually.

(v) Identification of the 350nm and 525nm Emission Components: The fluorophore of the 350nm and 525nm emission peaks in the embryonic spectra were identified. (a) Isolation, characterization, and identification of the 350nm emission component: As demonstrated in section

(ii) of this chapter, the 350nm emitting substance is found in the soluble-protein fraction of the embryonic homogenate. This emission peak (350nm) is characteristic of proteins which contain tyrosyl and tryptophyl residues (Gerard, et. al., 1975; Sasaki, 1975). In order to examine the possibility that the 350nm emission is due to a protein fluorescence, the following procedures were adopted.

In preliminary attempts to extract this fluorophore, the proteins in an embryonic homogenate were precipitated by addition of 98 percent ethanol to a final concentration of 75 percent as described in Chapter 2-3 (xiv). The majority of the fluorescence at 350nm was recovered in the ethanol-insoluble precipitate. An embryonic homogenate was also subjected to a chloroform extraction as described earlier, and the fluorescence at 350nm was primarily in the interface material (denatured protein layer). The response to these two extraction procedures suggests that the 350nm emission component is associated with a protein.

To test this hypothesis further, investigations were continued by examining the solubility characteristics and response to proteolytic digestion of the 350nm emission component in the soluble-protein fraction of the embryonic homogenate. The procedures utilized are described in section (iii) of this chapter and are outlined in Fig. 25. A portion of the soluble-protein fraction was precipitated with 5 percent TCA as described earlier. Nearly all of the fluorescence at 350nm was recovered in the TCA-insoluble material. This indicates that the component is acid-insoluble and coincides well with the behavior of neutral and acidic proteins. To test this further, a 1 mg portion of the TCA-insoluble material, suspended in 3 ml of Tris-HCl (pH 7.8), was digested with 100 μ g of α -chymotrypsin (in 10 μ l of water) by incubation at 37°C for 16 hours. After this digestion the TCA precipitation was repeated, and

Fig. 25. Flow chart outlining the experimental procedure utilized in characterization of the 350nm emission component obtained from the soluble fraction of a six-day embryo homogenate. The details of each step are presented in full in the text. (*Treatment with purified pronase, as described in text, yielded similar results).

Soluble Fraction (0.2 M fraction
D.S.G.)

- TCA (5% final volume)
- spun 32,000 g 10 min. (J-21)

Precipitate

Supernatant

- wash 5% TCA
- solubilize in alkali
- neutralize (final pH 7.8)
- all fluorescence at 350nm recovered here
- α - chymotrypsin (pronase)* digestion at 37°C for 16 hours
- TCA (final volume 5%)
- spun 32,000 g 10 min. (J-21)

- neutralize (final pH 7.8)
- no fluorescence at 350nm

Precipitate

Supernatant

- wash with 5% TCA
- solubilize in alkali
- neutralize (final pH 7.8)
- 1/4 of fluorescence at 350nm recovered here.

- neutralize (final pH 7.8)
- 3/4 of fluorescence at 350nm recovered here.

the fluorescence at 350nm was now found in the acid-soluble form, indicating that proteolytic digestion had released the fluorophore. In order to confirm that the change in solubility is associated with the digestion of protein, the digestion was repeated with 100 μ g of pronase, a non-specific protease. Similar results were obtained from this digestion. The pronase was less useful because of the fluorescence demonstrated by the enzyme solution itself, as noted in section (iii).

The results presented here indicate that the 350nm emission peak in the embryonic spectra is apparently due to the fluorescence of soluble proteins. The excitation and emission peaks coincide with peaks produced from solutions of purified protein containing tryptophane, as reported by Saski (1975). Therefore the spectral patterns were recorded from solutions of tyrosine and tryptophane as described in Chapter 2-3 (xii). The pattern obtained from the tryptophane solution (Fig. 26) is similar but not identical to that demonstrated by the 350nm emission component. The differences in the patterns is probably due to the fact that the fluorescence properties of the amino acid are changed upon incorporation into proteins (Gerard, *et. al.*, 1975; Sasaki, 1975). The 350nm emission component is precipitated by ethanol, chloroform, and TCA, all of which cause the precipitation of protein. Finally, the 350nm emission component is released by proteolytic digestion to a form soluble in TCA.

(b) Isolation, characterization, and identification of the 525 nm emission component: The spectral characteristics of the 525nm emission component in the embryonic homogenate are similar to those characteristic of the vitamin, riboflavin (Dudley, 1964). Therefore solutions of riboflavin, flavin mononucleotide, and flavin adenine dinucleotide were examined in buffer "B" (pH 7.8), as described in Chapter 2-3 (xii), and the spectral patterns obtained are seen in Fig. 27.

Fig. 26. Excitation and emission patterns obtained from solutions of L-tryptophane in buffer "B" at pH 7.8. There is a slight shift to the red in the excitation and emission peaks as concentration is increased from 0.01 mM (1A and 2A) to 0.5 mM (1B and 2B). (1A) represents the excitation spectrum for the emission peak at 355nm (2A); (1B) represents the excitation spectrum for the emission peak at 360nm (2B).

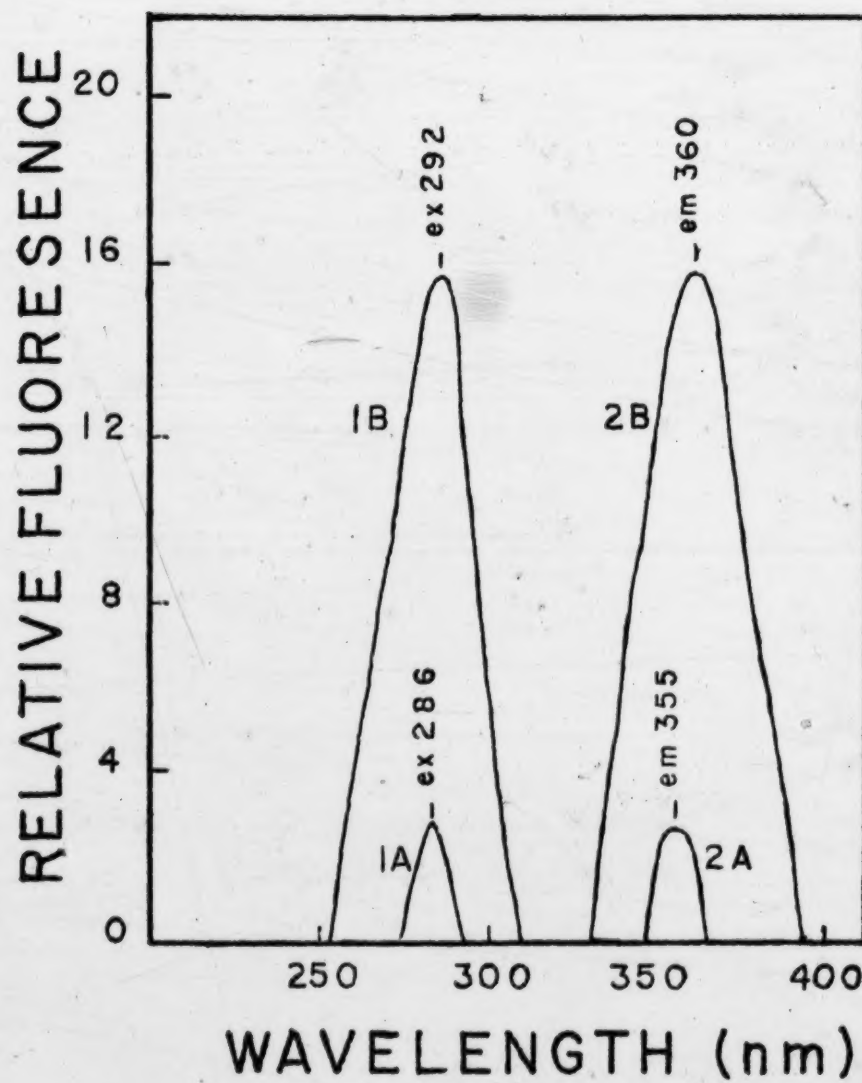
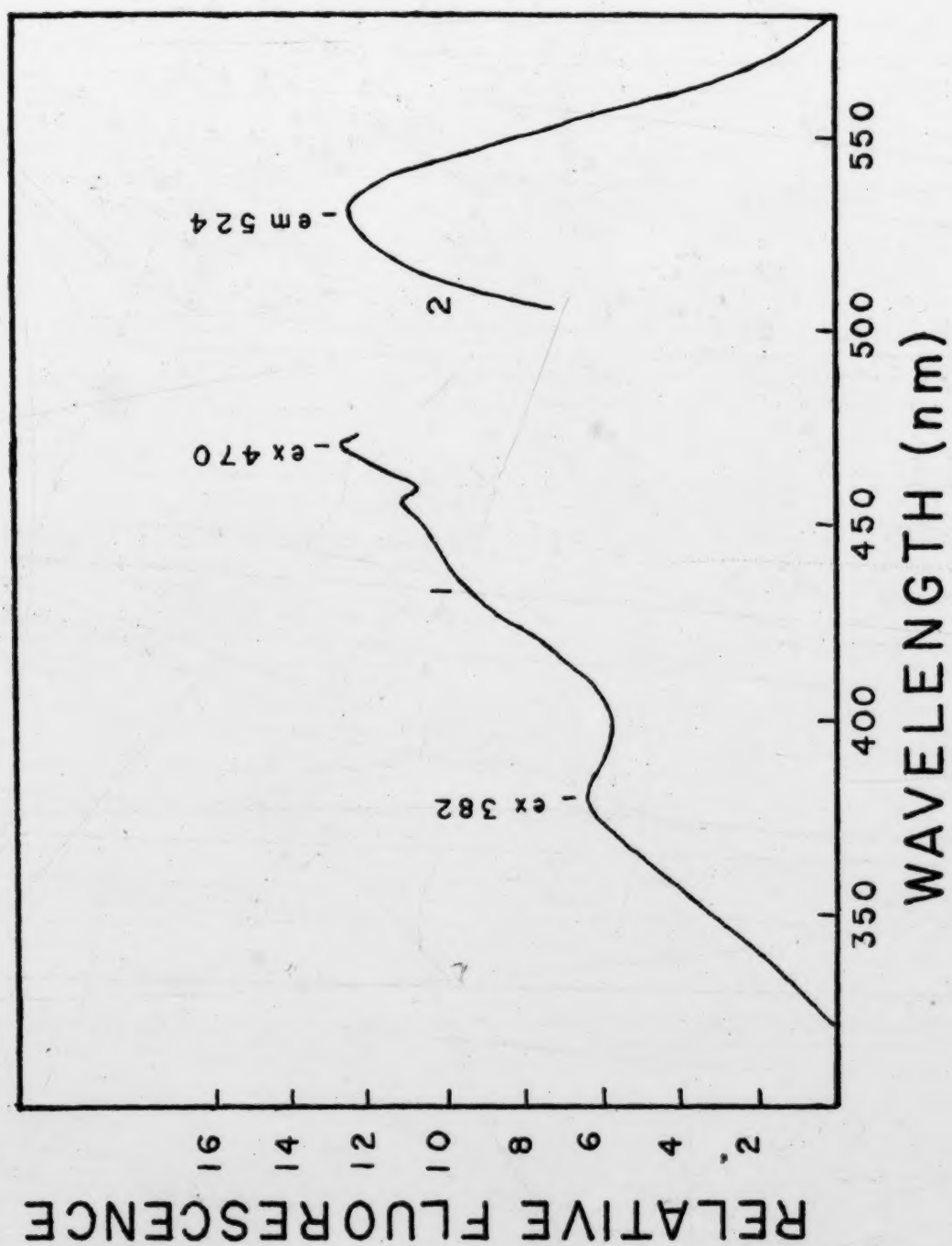


Fig. 27. Fluorescence pattern of a riboflavin solution in buffer "B" at pH 7.8. Equivalent concentrations of flavin mononucleotide and flavin adenine-dinucleotide demonstrate spectral patterns identical to this pattern; the former is at approximately the same level of fluorescence intensity as the riboflavin solution, and the latter is at a somewhat lower level of intensity for a given concentration.



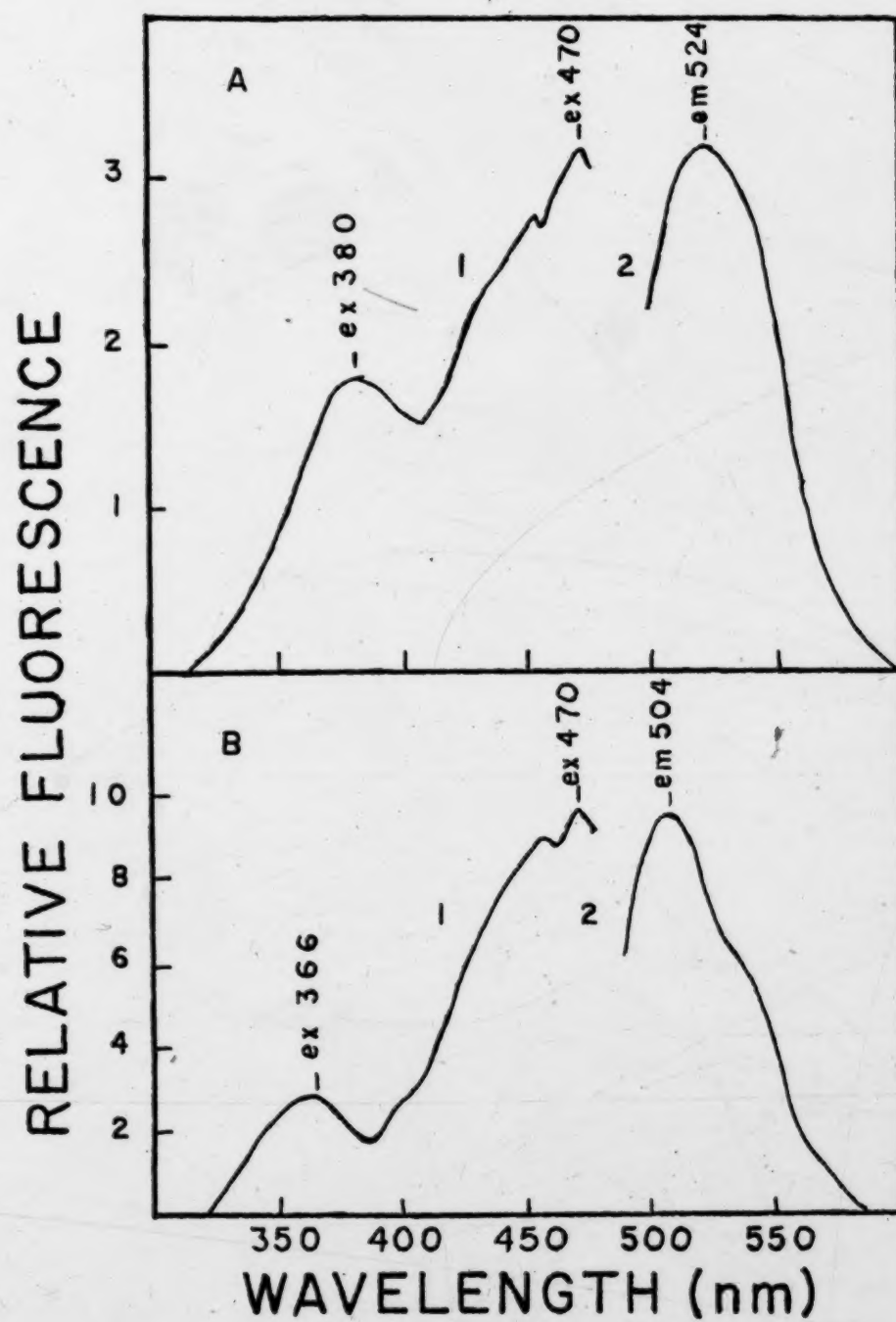
Upon investigation of its solubility properties, the 525nm emission component was found to be soluble in ethanol and TCA, but insoluble in chloroform. Riboflavin has similar solubility characteristics, being soluble in ethanol and acidic aqueous solutions but totally insoluble in chloroform, due to the D-ribose group on the isoalloxazine ring structure (Dudley, 1964). Upon exposure of an alkaline solution of riboflavin to ultraviolet irradiation, the D-ribose group is broken off, leaving a methyl group exposed. The water-soluble riboflavin is thus converted to a chloroform-soluble form lumiflavin, accompanied by a blue shift in the emission peak to 504nm (Dudley, 1964). This known behavior of riboflavin proved to be a valuable tool in the identification of the 525nm emission component found in the embryonic homogenate.

A 4ml portion of the post-nuclear supernatant from a six-day embryo was extracted four times with equal volumes of water-saturated chloroform as described earlier. These chloroform layers were combined, dried overnight, and suspended in buffer "B", (pH 7.8). This solution was examined, and only minor amounts of fluorescence were detected. The aqueous layer from the chloroform extraction was air dried to remove any residual chloroform and was found to contain the majority of the fluorescence at 525nm (Fig. 28A). This solution was then adjusted to pH 11 by titration with 0.1 N NaOH. It was irradiated with a Camay ultraviolet lamp (type TL-900, Muttez, Schwelz), at a distance of 14 cm for 45 minutes. After irradiation, the solution was brought to pH 10.5 by titration with 0.1 N HCl and was extracted with water-saturated chloroform. The chloroform layer contained a major emission peak at 540nm, excited at 366nm and 470nm (Fig. 28B). The aqueous layer contained very little fluorescence.

This behavior is identical to that demonstrated by a solution of

Fig. 28 (A). Fluorescence pattern of the buffer layer of the homogenate of a six-day embryo extracted four times with water-saturated chloroform. Minor emission peaks are seen at 350nm and 440-470nm. The excitation (1A) and the emission (2A) spectra are shown.

Fig. 28 (B). Fluorescence pattern from the chloroform layer obtained by re-extraction of the solution examined in 28 (A) after U V irradiation at alkaline pH. When a riboflavin solution was treated in a similar manner, identical results were obtained. The excitation (1B) and emission (2B) spectra are shown.



riboflavin when subjected to a similar treatment, converting the riboflavin to lumiflavin. Therefore it is possible to conclude that the 525nm emission peak in the embryonic homogenate is attributable to riboflavin or a derivative (*i.e.*, FAD or FMN). In order to determine which form of the vitamin is present in the embryo, further investigation is necessary.

5-3. Summary and Discussion:

The main objective of the experiments described in this chapter was to isolate, characterize, and identify the fluorophores involved in the pronounced spectral shift in the embryonic fluorescence spectrum described in Chapter 4. It was hoped that such an approach would enable us to determine the cause of this spectral shift, and also the role the substances play in cytological and/or morphological changes occurring at the time this shift is seen. As a spinoff of the experiments described in this chapter, evidence was obtained concerning the identity of the fluorophores responsible for the other two regions of emission found to dominate the embryonic spectra.

The ultraviolet emission peak at 350nm is apparently due to the fluorescence of aromatic amino acid residues in soluble protein. The increase in intensity at this peak, observed over the first week of development, evidently results from an increase in the soluble protein concentration during this period. The 525nm emission component was found to demonstrate solubility and fluorescence behavior characteristics of vitamin B₂ (riboflavin) and its derivatives, flavin mononucleotide and flavin adinine dinucleotide (for a review, see Koziol, 1971). Again, the increase in intensity of this emission peak over the developmental period under study here merely indicates an increase in vitamin B₂ content.

The function of this vitamin is well established. Its appearance at this time in development coincides with known increases in activity of a metabolic system, in which it is required as a co-factor (Brand, et. al., 1960).

In order to determine the sub-cellular location of the 430-440nm and 470nm emission substances, homogenates of three-day and six-day embryos were fractionated by differential centrifugation and ultracentrifugation procedures. This approach also served as the first step in purification of these fluorophores, separating them from nonfluorescent embryonic material. The majority of the fluorescence at 430-440nm and 470nm was recovered in the soluble-protein fraction of these homogenates, indicating that the fluorophores are either freely soluble in an aqueous medium or bound to a soluble "carrier" component.

Chromatographic techniques were then employed in an effort to purify the fluorophores, concentrating first on the isolation of the 470nm emission component in the soluble-protein fraction of a six-day embryo. These attempts were unsuccessful because the 470nm emission peak became unstable, and was lost, during the chromatographic separation. However, this instability proved to be useful in this investigation. Careful examination revealed that the 470nm emission peak shifts to a stable peak at 440nm upon exposure to a variety of conditions. This apparent reversal of the spectral shift suggested that a single type of fluorophore was involved in both 440nm and 470nm emitting substances. Therefore, by concentrating our investigation on the component responsible for the stable 440nm emission peak, we hoped to gain insight into the cause of the original spectral shift.

An examination of the solubility behavior of the 440nm emission substance, before and after specific enzymatic digestions, revealed

that this fluorophore is associated with a variety of binding components. In the soluble-protein fraction, the major type of binding component is apparently a lipoprotein or possibly a glycolipoprotein "carrier" molecule, although there are also minor amounts of a small peptide or low density lipoprotein "carrier". In the microsomal and lipid fractions the binding component appears to be lipid in nature, suggesting that a portion of this substance is membrane or matrix bound in the embryo.

Many biologically active molecules, such as hormones or fat soluble vitamins, are normally associated with specific binding molecules during storage and/or transport (e.g., vitamin A, Breman, 1973; Heller, 1976a). Because many of these "active" molecules are insoluble and unstable in aqueous solutions, the "carrier" molecules serve to stabilize and grant solubility to these substances during transport in the serum and when stored in the cytosol (Futterman, 1972; Heller, 1976b; Ong, 1974). Also binding to a "carrier" prevents these substances from exerting their biological effects non-specifically. Many such substances are toxic in a free form (Morris, 1974). The association of the 440nm emission substance with the variety of binding components noted above implies that this fluorophore is being transported within the embryo, and incorporated into cells and sub-cellular components, during the first week of development.

Due to the small size of the embryos examined in this investigation, we were unable to separate the serum-soluble components from the cytosol-soluble components. Therefore both are found in the soluble-protein fraction in this study. Because the 440nm emission substance is associated with several different components in this fraction, it is possible that this fluorophore is bound to both serum-soluble and cytosol-soluble components in the six-day embryo. The serum carrier would serve to

transport the fluorophore to a specific target cell or tissue, moving it from either the yolk, as suggested in Chapter 4, or from the site of production within the embryo itself. Upon incorporation into the receptor, the cytosol "carrier" component would bind and store the fluorophore until its use, or until it is incorporated into a specific organelle.

We had hoped to be able to identify this fluorophore and, possibly, examine the role it plays in developmental changes occurring at the time of the spectral shift. However, due to the instability of the fluorophore upon release from its binding component, we were unable to isolate this substance for identification. This must be the objective of further investigation. Although identification was not permitted, the observations reported in this chapter indicate that the shift in emission peak during development is apparently due to a change in the type of carrier to which the fluorophore is bound (e.g., the transfer from a serum "carrier" to a cytosol "carrier"). This may represent a general phenomenon occurring at the cellular level in the entire embryo, or it may be taking place at a specific target cell or tissue. In either case, the fluorometric examination described in this thesis has permitted the detection of a unique change at the molecular level, and has allowed us to pinpoint the precise time in development at which this change occurs.

In conclusion, as noted in Chapter 1, the mechanisms which control the process of differentiation and morphogenesis are not well understood. Factors in the extra-cellular environment are known to play an important role in the control and synchronization of these processes (Brunner, 1977). However, what these factors are and how they exert their effect is not known. Preliminary investigations by Lauber and Kato (personal communication), and by Igarashi (personal communication), had suggested that a

fluorometric examination of embryonic development might serve as a means of detecting factors involved in these processes. Therefore such an approach was undertaken in the investigation described in this thesis.

The results of the fluorometric examination described here indicate that there is a pronounced spectral shift in one of three emission peaks which dominate the embryonic spectra during the first week of development. This shift begins at Hamburger-and-Hamilton stage 22 and is completed by stage 25. Examination of the components responsible suggests that a single type of fluorophore is involved. The solubility characteristics of this fluorophore indicate that it is bound to serum, cytosol, and membrane components in the homogenate of a six-day embryo. The association with these components suggests that this fluorophore is the object of a serum transport system, and that the spectral shift starting at stage 22 may be caused by the transfer of the fluorophore from a serum "carrier" to a cellular "carrier". Upon binding to a different "carrier", the molecular environment of the fluorophore may be altered in a manner which affects the spectral characteristics of this fluorophore.

Unfortunately the fluorophore is rapidly decomposed upon release from its binding components, preventing its isolation and identification in this study. However, from the observations made here, it is suggested that isolation and identification might be accomplished by first purifying the "carrier" fluorophore complex. Once identification is accomplished and the solubility characteristics are reconfirmed, efforts might then be directed toward an histological search for the site within the embryo at which this shift is occurring. This accomplished, it would then be possible to examine the biological role which this compound plays in early embryonic development.

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